Cultivation of Microalgae: Lipid Production, Evaluation of Antioxidant Capacity and Modeling of Growth and Lipid Production

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Table of Contents

CHAPTER 1

Introduction

Microalgae biotechnology including bioprocess engineering and its bio products, has gained a great deal of attention in the last decade. Microalgae are aquatic unicellular photosynthetic microorganisms, which are able to absorb the sun's energy to fix water and CO_2 and release O_2 into the atmosphere. Microalgae provide many biotechnology applications in various industrial branches such as food, cosmetics, pharmaceuticals, energy and environmental industries. The application of microalgae offers a promising eco-friendly source of energy, although it still needs optimization to lower production costs and increase yields. Microalgae have been used successfully in environmental applications to remove inorganic and organic pollutants. Moreover, microalgae have been used to reduce the concentration of greenhouse gases in the atmosphere by converting carbon dioxide into biomass, which helps to solve the problem of global warming.

1.1. Microalgae diversity

Microalgae can be found in salt water, brackish water, freshwater and snow habitats. There is a diverse range of microalgae body types including coccoids, coenobial colonies, flagellate colonies, as well as branched and unbranched filaments. With an estimated 36,000 – 10 million species, algae are the major contributors to biodiversity [1]. Each species is unique and plays a role in the ecosystem. The relationships between the three major lineages of life, archaea, bacteria and eukaryotes are described in Fig. 1-1.

Microalgae have been categorized into ten groups, which often contain a reference to the color of the microalgae included in them: Cyanobacteria, blue-green algae; Chlorophyta, green algae; Rhodophyta, red algae; Glaucophyta; Euglenophyta; Haptophyta; Cryptophyta; photosynthetic Stramenopiles; Dinophyta; and Chlorarachniophyta [1].

Blue-green algae (Cyanobacteria) are much closer to bacteria in terms of structure and their cells lack a nucleus and chloroplast. Cyanobacteria can be found in ocean, soil, mountain, hot-spring and snow habitats. They are also known as a source of chlorophyll a, phycocyanin, phycoerythrin, xantophyll and ß-carotene. A number of

cyanobacteria are known to fix nitrogen gas and release it increasing the fertility of soil and water. Cyanobacteria species contain potential products for medicinal [2] and energy applications [3]. Some of this group has secondary metabolites that can potentially be used as therapeutic agents, such as antivirals, immunomodulators, inhibitors and cytostastics [4, 5].

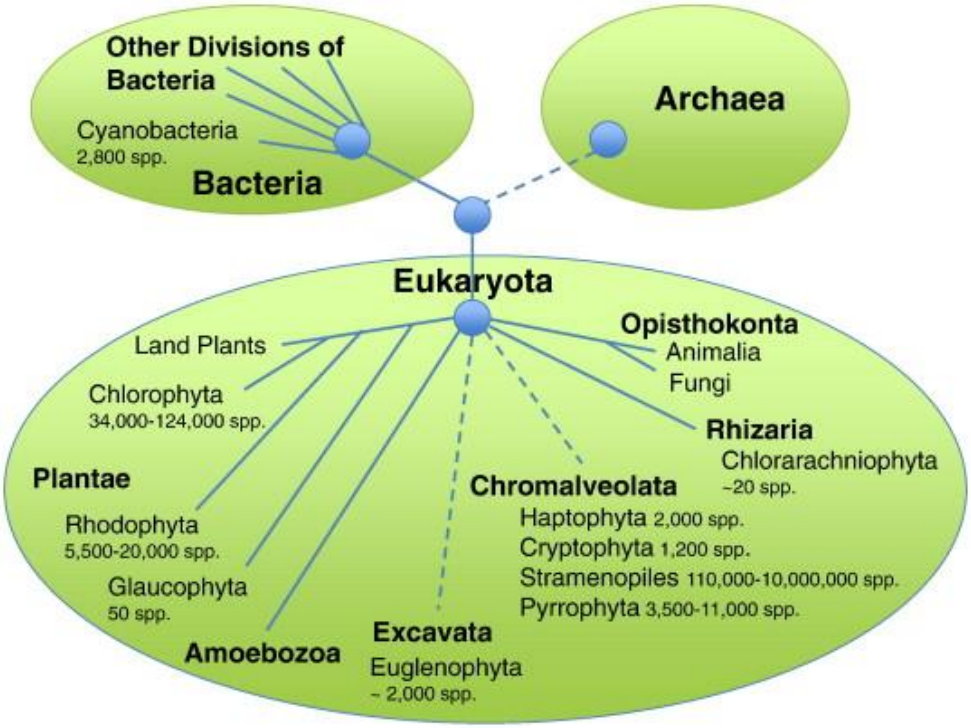


Fig. 1-1. Relationships between the three major lineages of life, archaea, bacteria and eukaryotes [6, 7].

Most green algae (Chlorophyta) are found abundantly especially in freshwater, but also in marine and brackish environments. Green algae contain chlorophylls a and b, xanthophyll, ß-carotene, lutein and lycopene [1]. The storage product is starch, which is located in their plastids. Some species can accumulate a high content of carotenoids under certain conditions. Red algae (Rhodophyta) can be found in marine, freshwater and terrestrial areas. The group accumulates starch in the cytosol and is known as a source of chlorophyll a, chlorophyll d, ß-carotene and zeaxanthin. A red microalga *Porphyridium* contains a high amount of arachidonic acid, pigments (phycoerythrin and phycocyanin) and extracellular polysaccharides [8]. Glaucophyta are a small group of freshwater microalgae with chlorophyll (a, b, c) and the phycobilin, phycocyanin and allophycocyanin, as well as ß-carotene as pigment accessories [1]. Euglenoids (Euglenophyta) are commonly found in freshwater, but some genera can also be found in marine habitats. They generally have two flagella and the cells contain chlorophyll a

and chlorophyll b. The storage product is a carbohydrate called ß-1,3 polyglucan (paramylon) found in cytoplasm [1].

Haptophyta algae are mostly found in marine habitats, but the species also inhabit freshwater and terrestrial environments. Many species of Haptophyta are flagella and most of them are unicellular. Haptophyta algae have an important role in both global biogeochemistry and in the food webs in natural and aquaculture systems. Chrysolaminarin (ß-1,3-polyglucan) is their primary storage product. The cells contain the pigment fucoxanthin, diadinoxanthin, ß-carotene, chlorophyll a, as well as chlorophyll c [1]. Most Haptophyta excrete extracellular polysaccharides, e.g., up to 64% of total polysaccharides in *Phaeocytis pouchetii* [8]. This group is considered to be a high-quality food for marine zooplankton, because a certain genus contains high-level polyunsaturated fatty acids (PUFA). PUFA such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are used in aquaculture systems or bioreactor systems for the industrial production of omega-3 fatty acids [9]. Cryptophyta algae are relatively small flagellates up to 50 um in length. Cryptophyta are also a high-quality food source for zooplankton, which is important in both natural systems and aquaculture. Photosynthetic pigments of Cryptophyta include phycoerythrin, phycocyanin, α- and ß-carotene, xanthophyll, and chlorophyll a and c. Starch and lipid are storage products [1].

Stramenopiles (Heterokonta) consist of brown algae (Phaeophyta), yellow-green algae (Xanthophyta), golden algae (Chrysophyta) and diatoms (Bacillariophyta). They are referred to as “brown line” and found in marine, terrestrial and freshwater habitats. The diatoms represent the largest group in this division. The storage products are chrysolaminarin, carbohydrates and lipids. Chrysolaminarin consists of 20-60 glucose units with ß-1,3 linkages and some ß-1,6-linked branches. The pigment accessories are ß-carotene, fucoxanthin, vaucheriaxanthin as well as chlorophyll a and chlorophyll c. In addition, ß-carotene is the major yellow accessory pigment in this group. Fucoxanthin delivers golden-brown or brown pigmentation to diatoms, chrysophyceans, brown algae and some others. Vaucheriaxanthin is a dominant accessory pigment in raphidophyceans, eustigmatophyceans, and xantophyceans which tend to have yellow-green or yellow-brown pigment [1].

Pyrrophyta (Dinophyta) can be found in fresh or brackish waters, coastal tropical and subtropical oceans, water and seawater. Oil and starch are storage products; the group contains unique sterols, fatty acids (DHA) and other metabolites [1]. Most Dinophyta are photosynthetic or heterotrophic unicellular form with two distinctive

flagella. Dinophyta species deliver a golden-brown color as a unique accessory xanthophyll pigment, called peridinin [1]. Chlorarachniophyta are distributed in small groups in marine environments, e.g., coastal regions and open ocean waters [10] and they possess various cell types such as amoeboid, coccoid and flagellate. The major pigments are chlorophyll a and b [1].

1.2. Chemical composition of microalgae

The major chemical components of microalgae are carbohydrates, proteins and lipids. Table 1-1 provides an overview of their chemical composition [11].

Table 1-1. The chemical composition of different algae biomass (% of dry weight) [11]

Algae	Protein	Carbohydrate	Lipid
Anabaena cylindrical	43-56	25-30	4-7
Aphanizomenon flos-aquae	62	23	3
Clamydomonas reinhardtii	48	17	21
Chlorella pyrenoidosa	57	26	2
Chlorella vulgaris	51-58	12-17	14-22
Dunaliella salina	57	32	6
Euglena gracilis	39-61	14-18	14-20
Porphyridium cruentum	28-39	40-57	9-14
Scenedesmus obliquus	50-56	10-17	12-14
Spirogyra sp.	6-20	33-64	11-21
Spirulina maxima	60-71	13-16	6-7
Spirulina platensis	46-63	8-14	4-9
Synechococcus sp.	63	15	11

Starch, sugars, glucose and other polysaccharides are forms of carbohydrate, which are found in up to 60% of dry weight in the microalgae. Microalgae contain a high level of protein up to 70% of their dry weight [11]; this is the main reason for the application of microalgae as a protein source. Microalgae lipids are composed of saturated or unsaturated fatty acids and the average lipid content varies between 2 and 21% of dry weight (Table 1-1), but some species are capable of depositing up to 90% of their dry weight under stress conditions [12]. Microalgae also represent a valuable

source of polyunsaturated fatty acids, vitamins, antioxidants, pigments, as well as specials products such as toxins and isotopes [8].

1.3. Microalgae products and applications

There is a wide range of microalgae products that can be harvested from the microalgae biomass. The quality and type of these products depends on microalgae species, culture condition, and recovery methods. The products of microalgae can be divided into three categories: 1) biomass 2) food and bulk chemicals: protein, carbohydrates, alcohol, oils and fatty acids, etc.; 3) special chemicals: pigments, vitamins, volatile compounds, etc.

Table 1-2. Market estimates for microalgae products [8]

Product group	Product	Retail value (US. $x10^6)	Development
Biomass	Health food	1,250-2,500	Growing
	Functional food	800	Growing
	Feed additive	300	Fast-growing
	Aquaculture	700	Fast-growing
	Soil conditioner		Promising
	Biofuel	1,300*	Fast growing
Colorants	Astaxanthin	<150	Starting
	Phycocyanin	>10	Stagnant
	Phycoerythrin	>2	Stagnant
Antioxidants	ß-carotene	>280	Promising
	Tocopherol		Stagnant
	Antioxidant extract	100-150	
	ARA	20	Growing
	DHA	1,500	Fast-growing
	PUFAs extract	10	
Special products	Toxin	1-3	
	Isotopes	>5	

*http://westernfarmpress.com/markets/algae-based-biofuels-billion-dollar-market opportunity
ARA: Arachidonic fatty acid
DHA: Docohexaenoic fatty acid

Table 1-2 shows a review of the main products and market estimates of microalgae products in recent years. The main microalgae market applications are human and animal nutrition, cosmetics and research. Currently, fewer than 20 microalgae species are used in industry and the algae mostly produced are *Chlorella*, *Cryptecodinium cohnii*, *Dunaliella salina*, *Haematococcus pluvialis*, *Ulkenia* sp.,

Spirulina, Aphanizomenon flos-aquae, Odontella aurita, S*chizochytrium, Porphyridium cruentum*, *Phaeodactylum,* and *Nostoc*.

1.3.1. Biofuels

Microalgae are considered an alternative future energy source such as biodiesel, bio-ethanol, bio-methane, and bio-hydrogen (Fig. 1-2). The production of biofuels using microalgae provides some advantages compared to terrestrial plants. Microalgae 1) offer less competition with food crops, 2) require less water than land plants, 3) are able to use waste water for nutrient intake, 4) have a rapid growth rate, and 5) have greater photosynthetic efficiency [13]. On the other hand, the disadvantages of microalgae farming are the low biomass and when the algae cells are quite small, relatively high costs in algae harvesting.

Biodiesel is obtained from plant/ animal oil, which consists of triglycerides that are reacted with methanol using an acid / base/ enzymes as catalyst. This reaction produces glycerol and fatty acid methyl esters (FAME); the reaction is called transesterification. Microalgae for biodiesel have been reviewed by some researchers [13-15]. The important point for microalgae biodiesel replacing fossil fuels is that microalgae biodiesel should have lower production costs [16]. Microalgae oils contain different kinds of lipids, including saturated and unsaturated lipids. The saturated lipids in microalgae oil are very suitable for making biodiesel [17]. However, unsaturated lipids in microalgae oil can be reduced by partial catalytic hydrogenation [18, 19] in order to meet the standard criteria of microalgae biodiesel based on the ASTM Biodiesel Standard D 6751, Standard EN 14214 for vehicle use and Standard EN 14213 for heating oil [20]. Fossil oil could be completely replaced by microalgae oil in the future, but this will be dependent on the price of crude oil [21]. Today, many companies are interested in investing in making fuel from algae. About 200 companies around the world produce fuel from algae with various culturing methods; 75 companies have made significant investments and commitments. Algae companies can be found in the USA, Europe and Asia and numerous companies have made fast progress in turning biodiesel production into an economic process such as Cellana, Exon-Mobil, Origin Oil, PetroAlgae, Solazyme, etc. The cost for algae biodiesel is $ 6-18/ gallon and this lower cost can only be achieved through the use of open pond systems. The price, however, is still 2-8 times the price of non-algae biofuels and 3-9 times the price of fossil oil [22].

Currently, bioethanol is produced by fermentation of sugarcane. It has been known that microalgae cells contain carbohydrates and they can be hydrolyzed and fermented into ethanol. Microalgae are a promising source of bioethanol production. A number of microalgae species are able to produce a high content of carbohydrates. These species are good candidates for bioethanol production, for example, *Enteromorpha* contains up to 70% carbohydrate of dry weight, *Chlorella vulgaris* contains up to 37% starch of dry weight [23], and *Chlorella* accumulates up to 70% starch of dry weight [24]. Cultures of *Chlorococcum sp.* [25, 26] and *Chlamydomonas* [27, 28] are also candidates for bioethanol feedstock production.

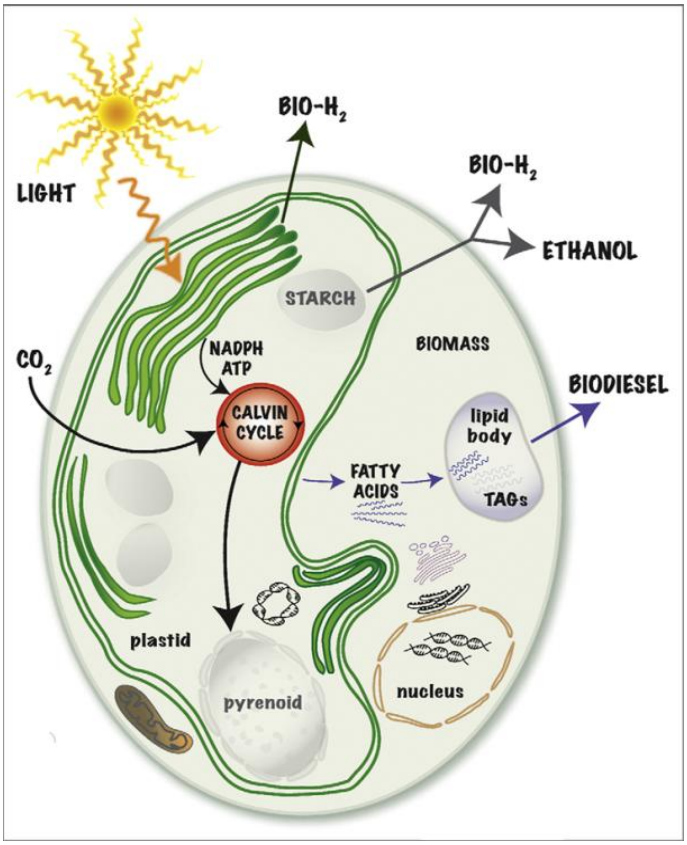


Fig. 1-2. Potential pathway from microalgae to fuels [29]

In addition to bioethanol, biodiesel, and biomethane, hydrogen is a source of clean energy. Hydrogen is produced from fermentation of biomass, photolysis of water, natural gas, coal, solar radiation, and wind. Biohydrogen can be produced from microalgae species to generate electricity [30-33]. *Chlamydomonas reinhardtii* has a potential for use in biotechnology with regard to hydrogen production. Today´s research into *Chlamydomonas reinhardtii* is focused on improving hydrogen production via genetic manipulation [34, 35]. The advantages of biohydrogen as a fuel are its eco-

friendly nature, efficient renewability, and the absence of CO_2 emissions during production and utilization [36].

Biogas is composed of methane (55-75%), carbon dioxide (25-25%) and other compounds (N_2, H_2S, H_2, and O_2 – less than 10%). The production of methane is processed by anaerobic digestion; afterwards the residual biomass could be processed for fertilizer. Methane can be produced from algal biomass or a residual of algae biomass in the range 0.29 - 0.59 $m^3\ kg^{-1}$ [37, 38].

1.3.2. Food, nutraceuticals and cosmeceuticals

Polyunsaturated fatty acids (PUFA) are carboxylic acids with long unsaturated hydrocarbon chains (≥ 18 carbon). PUFA are essential nutrients and must be provided from an external source. It is well known that PUFA are interesting fatty acids including n−6 (ω-6) and n−3 (ω-3) since they have health effects on human body. Omega-3 acids, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have potential applications in foods, foods supplements and aquaculture [39]. Sources of omega-3 are commonly fish and fish oil, but these are accompanied by some problems such as an unpleasant taste and poor oxidative stability [40]. Due to this, microalgae PUFA have been considered as a source of PUFA; more specifically, PUFA which are accumulated in fish originating from microalgae consumed at marine sites. A number of microalgae species have been produced, including DHA [41, 42] and EPA [43, 44]. Omega-6, such as γ-linolenic acid (GLA) and arachidonic acid (ARA), is also an interesting microalgae PUFA. GLA is produced by *Spirulina* [45, 46] and ARA is obtained from *Porphyridium* [47, 48].

Microalgae have been used to produce natural pigments (e.g., chlorophyll, carotenoids and phycobilins), which exhibit colors ranging from yellow, green, brown to red. Most algae contain these pigments up to 4% of dry weight under optimum conditions. Chlorophyll has been used in the food industry for coloring [49]. Microalgae carotenoids have commercial applications in cosmetics, food, pharmaceuticals and animal feed additives [50]. Microalgae produce several commercial carotenoids such as ß-carotene, astaxanthin, lutein, canthaxanthin, zeaxanthin, lycopene and bixin. The major phycobilins including phycoerythrin and phycocyanin are used as colorants in the food and cosmetics industry. Moreover, phycobilins are used for diagnostic purposes as fluorescent markers [51, 52]. The other pigment is named marennine; it is blue-green and synthesized by the marine diatom *Haslea ostrearia* which grows along the Atlantic

coast in France. According to Pouvreau et al. [53], marennine showed higher antioxidative and free radical scavenging activities than natural and synthetic antioxidants commonly used in food. Therefore, marennine has a potential use as a natural antioxidant and also for food coloring [53]. The production of marennine on an industrial scale is not yet available; today´s research is focused on refining it.

Nutraceuticals are a union between nutrition and pharmaceutics; they contribute to human health [54]. Bioactive ingredients which include nutraceuticals are fatty acids, carotenoids, sphingolipids, glycerolipids, vitamins and sterols. Microalgae are a good candidate for drugs to treat cancer [55], HIV [56] and herpes simplex virus [57]. Some bioactive compounds have possible health effects; these include carotenoids such as antioxidant antivirals, antimicrobials, and antiproliferatives [58, 59]; and glycerolipids such as antimicrobials and anti-inflammatories [60, 61]. Vitamin E [62, 63] and polyunsaturated fatty acids including DHA, EPA, GLA, and ARA have positive a health effect by reducing the risk of certain heart diseases [56, 64, 65] and as an anti-inflammatory [65]. Gamma linolenic acid (GLA) is medically important since in the human body it is converted into arachidonic acid (ARA) and then into prostaglandins [66].

Cosmeceuticals are cosmetic products that contain active ingredients which enhance or protect the appearance or condition of the human body such as anti-aging creams and moisturizers [67]. Microalgae extracts are used mostly in face and skin care products, e.g., anti-aging cream, regenerating skin cream, emollient products, anti-irritant products, sun protection cream and hair care products. Research regarding the enhancement of youthful-appearing skin is growing very rapidly. A number of cosmetic companies have produced their own microalgae to use algae in their products. Dermochlorella® is produced by Codif France. It uses *Chlorella vulgaris* as a natural carotenoid source and claims to stimulate collagen synthesis in skin, regenerate tissue and reduce wrinkles. Protulines® is manufactured by the cosmetic company Exsymol (Monte Carlo, Monaco) and uses *Arthrospira* containing gamma-linolenic acid (GLA) that serves as an active ingredient to repair the effects of aging skin. Pentapharm Ltd. (Switzerland) produces PEPHA®–TIGHT using elements of *Nannochloropsis oculata* (i.e. polysaccharides, amino acids and vitamins) and claims that it has excellent skin-tightening properties. PEPHA®-Ctive uses *Dunaliella salina* which is rich in β-carotene; this product has the ability to significantly stimulate cell proliferation and turnover and positively influence the energy metabolism of skin [39, 68]. Other anti-aging products

use microalgae such as Skinicer® (Ocean Pharma, Germany), which uses *Arthrospira maxima*; AlguardTM (Frutarom, Israel), which uses *Porphyridium* sp.; Photosomes (Estèe Lauder, USA), which uses *Anacystis nidulans*; Algenist (Solazyme, USA), which uses alguronic acid extracts from microalgae; and Phytomer (Phytomer, France), which uses marine microalgae. Moreover, several studies reported the use of biologically active ingredients from microalgae extracts for potential applications in nutraceuticals, cosmetics and pharmaceutical formulations including terpenoids [69], astaxanthin [70], polysaccharide [71], phycoerythrin [72] mycosporine-like amino acids (MAAs) [73, 74], and phenolic compounds [75].

1.3.3. Animal feed and aquaculture

Microalgae are important in animal feed and aquaculture species as a food source. The main applications of microalgae biomass in aquaculture are as follows: live feed for the larvae, fish species and zooplankton [76]; food additive to supply basic nutrients, enhance the color of salmonids or for other biological activities [77]; stabilization and improvement of quality of culture medium [78]; stimulation of the immune systems of fish and animals [39]; and probiotic effects [79]. About 40 species of microalgae are used in aquaculture worldwide; the microalgae used most in aquaculture are *Spirulina, Chlorella, Scenedesmus, Dunaliella*, *Tetraselmis, Isochrysis, Pavlova, Skeletonema, Chaetoceros, Phaeodactylum, Nitzschia, and Thalassiosira* [76].

1.3.4. Environmental and agriculture

Rapid industrialization has had a severe negative impact on the environment. It is of grave importance to reverse the damage caused by industrialization by implementing an eco-friendly system using microorganisms. The use of microorganisms in wastewater treatment has attracted the attention of researchers all over the world. Microalgae have also been successfully used in bioremediation. Some articles have reported the use of microalgae to eliminate contamination caused by nitrogen and phosphorus. For example, microalgae are grown in fermented swine wastewater [80], dairy manure effluent [81, 82], carpet mill effluent [83], human urine [84], flue gas [85, 86] and wastewater [87, 88]. Moreover, some immobilized microalgae species have been shown to remove nitrogen or ammonium from waste water [89, 90]. Microalgae species are able to remove heavy metals [91, 92], and organic pollutants [93, 94].

Bio-mitigation using microalgae is receiving increased attention due to rapidly increasing global warming as a result of the increase of CO_2 in the atmosphere. Mitigation of CO_2 using microalgae offers several advantages: it elevates CO_2 levels, and produces high-value bioproducts and biofuels [95, 96]. It has been calculated that one kilogram of dry alga biomass utilizes about 1.83 kg of CO_2 [16]. A number of microalgae have been shown to be able to fix CO_2 from industrial waste for growth, for example: Yun, et *al*. [97] reported that *Chlorella vulgaris* sequesters 0.624 g CO_2 L^{-1} in steel plant wastewater. *Chlorella* sp. reduces 10-50% of CO_2 concentration in flue gases [98]. *Scenedesmus* sp. is able to grow in flue gas with a high rate of biomass production (0.218 g L^{-1} per day) [99].

Microalgae show promise as soil fertilizer products and the inoculation of microalgae to increase the fertility of soils has been successfully attempted. For example, nitrogen-fixing blue-green algae integrated with wastewater treatment could be converted into organic nitrogen fertilizer [100, 101], e.g., *Anabaena* and *Nostoc* are used as nitrogen fertilizers for rice production [102, 103], and future research might be steered towards combatting plant diseases caused by bacteria or viruses.

1.4. Research objectives

Tropical microalgae have potential for biotechnology applications, but are still largely unexplored as a natural resource and have not yet been explored scientifically. Because of their diversity, tropical microalgae offer great potential for innovation in energy, environmental applications, human and animal nutrition, and cosmetics. Two tropical microalgae, *Chlorococcum* sp. and *Nannochloropsis* sp., are used in this study. The major aims of this study were:

- To investigate the effect of environmental parameters including light intensity, nitrate concentration, salinity, acetate concentration as well as the influence of culture age on biomass and lipid production of indigenous tropical freshwater microalga *Chlorococcum* sp.
- To investigate the effect of environmental parameters such as nitrate concentration, acetate concentration and crude glycerol concentration as well as culture age on biomass and lipid production of the indigenous tropical seawater microalga *Nannochloropsis* sp.
- To evaluate of the antioxidant activity in both microalgae and the correlation of antioxidant capacity, total phenolic and total carotenoid content.
- To study a mathematical model that integrates biomass production, lipid accumulation and substrate consumption. The model was used to estimate the optimum values of parameters that have the most influence in the production process.

CHAPTER 2

Microalgae Production

2.1. Requirements of microalgae growth

Microalgae require some mineral nutrients for growth in addition to carbon, water, and light. The nutrients commonly required by algae are carbon, nitrogen, phosphorus and other essential nutrients such as sulfur, iron, magnesium, etc. [1].

2.1.1. Carbon

Carbon dioxide, inorganic carbon (e.g., $NaHCO_3$ and Na_2CO_3) and organic carbon (such as sodium acetate, glucose, glycerol, etc.) are carbon sources for microalgae growth. Air from the atmosphere and emissions from industrial power plants are also used as sources of carbon. Air from the atmosphere contains about 0.04% (v/v) carbon dioxide, and emissions from industrial source up to 15% (v/v) CO_2. Some microalgae species are able to use high concentrations of CO_2 for their growth. For example, *Chlorococcum littorale* has a tolerance of up to 40% (v/v) CO_2 [96, 104]. Some marine microalgae are able to use carbonates as a carbon source for their growth [105]. Organic carbon sources including glucose, acetate, glycerol, etc., are commonly used for microalgae cultivation [106, 107]. The use of CO_2 from power plant flue gas for microalgae cultivation was reported [98, 108, 109]. Moreover, the fixation of carbon dioxide as a carbon source in microalgae cultivation has both economic and environmental value as it reduces the effects of global warming.

2.1.2. Nitrogen and phosphorus

Nitrogen is utilized to synthesize amino acids, nucleotides, chlorophylls and phycobilins. The common nitrogen sources are nitrate, ammonia and urea [107, 110]. Some algae are able to take up organic nitrogen such as yeast extract and glycine [107]. Algae can use nitrate and ammonium ions directly from surrounding water. Ammonium can be used more directly than nitrate to synthesize cellular N-compounds, and algae are able to convert nitrate to ammonium by using the enzyme nitrate reductase [1]. Wastewater, which contains large concentrations of nitrogen, is a cheap source of nitrogen. Phosphorus is an essential nutrient which plays an important role in the

metabolism process, (ATP, DNA, RNA and phospholipids). Phosphorus is commonly found in the form of $H_2PO_4^-$ or HPO_4^{2-} and the cheapest phosphorus source comes from wastewater.

2.1.3. Other nutrients

Other nutrients are sulfur, iron, magnesium, silicon, as well as other trace elements. Iron is required as a cofactor for many enzymes, namely cytochromes, ferredoxins, catalases, glutamate synthetases, nitrogenases, nitrates, and nitrite reductases. Sulfur is needed for the biosynthesis of some amino acids, e.g., cysteine and methionine; some thylakoids in lipids, carrageenan, agar, biotin, dimethylsulfoniopropionate (DMSP); and also as cofactor for the enzymes nitrogenase and CoA. Magnesium is required for the biosynthesis of chlorophyll and other trace minerals and serves as a cofactor of many enzymes [1].

2.1.4. Environmental factors

Operational parameters in algae cultivation will be required such as light intensity, temperature, and pH value. Microalgae need a carbon supply and light to carry out photosynthesis to produce biomass. The light spectrum and intensity are major factors in the productivity and growth of microalgae. Eight photons of photosynthetically active radiation (PAR) (~48% of the incident of solar flux) are required to fix one molecule of CO_2 in carbohydrate, resulting in a maximum photosynthetic efficiency of about 9% [111, 112]. The photosynthetic efficiency is in the range of 4.5 - 7% under low to moderate light levels in pond and closed systems [113, 114]; this translates to a yield of 30-40 gram dry weight m^{-2} d^{-1} [112]. Due the high cost of installing and operating artificial light sources in photobioreactors, several investigations have looked into finding an efficient and cost-effective means of light production such as the application of different types of illumination to enhance the microalgae production rate and lipid content for use in biodiesel [115]. Fluorescent lamps are commonly used in laboratories that have high power consumption per unit PAR and high capital cost. Most commercial microalgae cultivations are still carried out in open ponds using the sun as a light source. In any case, microalgae cultivation in open ponds suffers from poor illumination and needs to increase light efficiency; using solar light collector could be a solution here [116].

Most algae are able to grow at temperatures between 20 and 24°C. However, the optimal growth temperature varies by species, strain culture and composition of the culture medium. Cultured strains usually tolerate temperatures in the range 16 to 27°C, but temperature tolerance is also found in some algae species, for example, strains from hot spring areas. Temperature is a sensitive parameter of algae regarding growth and metabolic activities. The effect of temperature on growth, chemical composition, fatty acid composition and lipid content was investigated by Converti et *al.* [117], Hoffman et *al.* [118], Renaud et *al.* [119], and Xin et *al.* [120].

Algae growth is influenced by pH value; the pH range for cultured algae is between 7 and 9. The optimal pH, however, depends on the algae strain and composition of the medium; extreme pH levels potentially have direct physiological effects. There are algae strains with optimum growth between pH 2 and 3, such as *Cyanidium caldarium* and *Galdieria sulphuraria*. Two factors can change the pH of algae culture i.e., the bubbling of the culture with CO_2-enriched air and the unequal cellular absorption of cation and anion.

2.2. Production system

There are two basic systems for microalgae production: open pond and closed (photobioreactors). Table 2-1 describes the advantages and limitations of various culture systems for microalgae.

2.2.1. Open ponds

The cultivation of microalgae in open pond systems is generally used in algae farming because of its relatively low cost and its simple configuration. Open ponds can be divided into natural waters (lakes, lagoons, ponds) and artificial ponds. Currently, four open pond systems are used in microalgae farming, namely raceway ponds, shallow big ponds, circular ponds and paddle wheel ponds. The application of open ponds for microalgae production brings with it a high risk of contamination from other microorganisms (e.g., bacteria, fungi or predators). Therefore, only some microalgae species can be cultivated in open systems, for example: *Dunaliella* with high salt tolerance, *Spirulina* with high pH tolerance and *Chlorella* with high nutrition demands [121]. The operating parameters of open pond systems are not easy to control because the effect of the local climate will have a large impact, such as temperature, light intensity, wind and others.

2.2.2. Closed systems: Photobioreactors

Microalgae can be grown in close systems under photoautotrophic, mixotrophic or heterotrophic conditions. Closed systems (photobioreactors) are easier to control and monitor compared to open pond systems, but the operating costs are much higher. Closed systems have several advantages, such as temperature control, pH control, high productivity, and no contamination from other microorganisms. Nowadays, research is focused on developing photobioreactor designs. There are three basic types of photobioreactor: tubular, flat plate and vertical column. Tubular and flat plate photobioreactors are commonly selected [122].

Table 2-1. Advantages and limitations of various culture systems for microalgae [123].

Systems	Advantages	Limitations
Open ponds	Relatively cheap, easy to clean after cultivation, good for mass cultivation of algae	Little control of culture condition, difficult to grow algae cultures for long periods, poor productivity, occupy large land area, limited to a few strains of algae, cultures are easily contaminated
Tubular photobioreactors	Large illumination surface area, suitable for outdoor cultures, fairly good biomass productivity, relatively cheap	Gradients of pH, dissolved oxygen and CO_2 along the tubes, fouling, some degree of wall growth, requires a large area of land
Flat-plate photobioreactors	Large illumination surface area, suitable for outdoor cultures, good immobilization of algae, good light path, good biomass productivity, relatively cheap, easy to clean, readily tempered, low oxygen build-up	Scale-up requires many compartments and support materials, difficulty in controlling culture temperature, some degree of wall growth, possibility of hydrodynamic stress to some algae strain
Vertical-column photobioreactors	High mass transfer, good mixing with low shear stress, low energy consumption, high potential for scalability, easy to sterilize, readily tempered, good for immobilization of algae, reduced photo-inhibition and photo-oxidation	Small illumination surface area, construction requires sophisticated materials, shear to stress to algae cultures, decrease of illumination surface area upon scale-up.

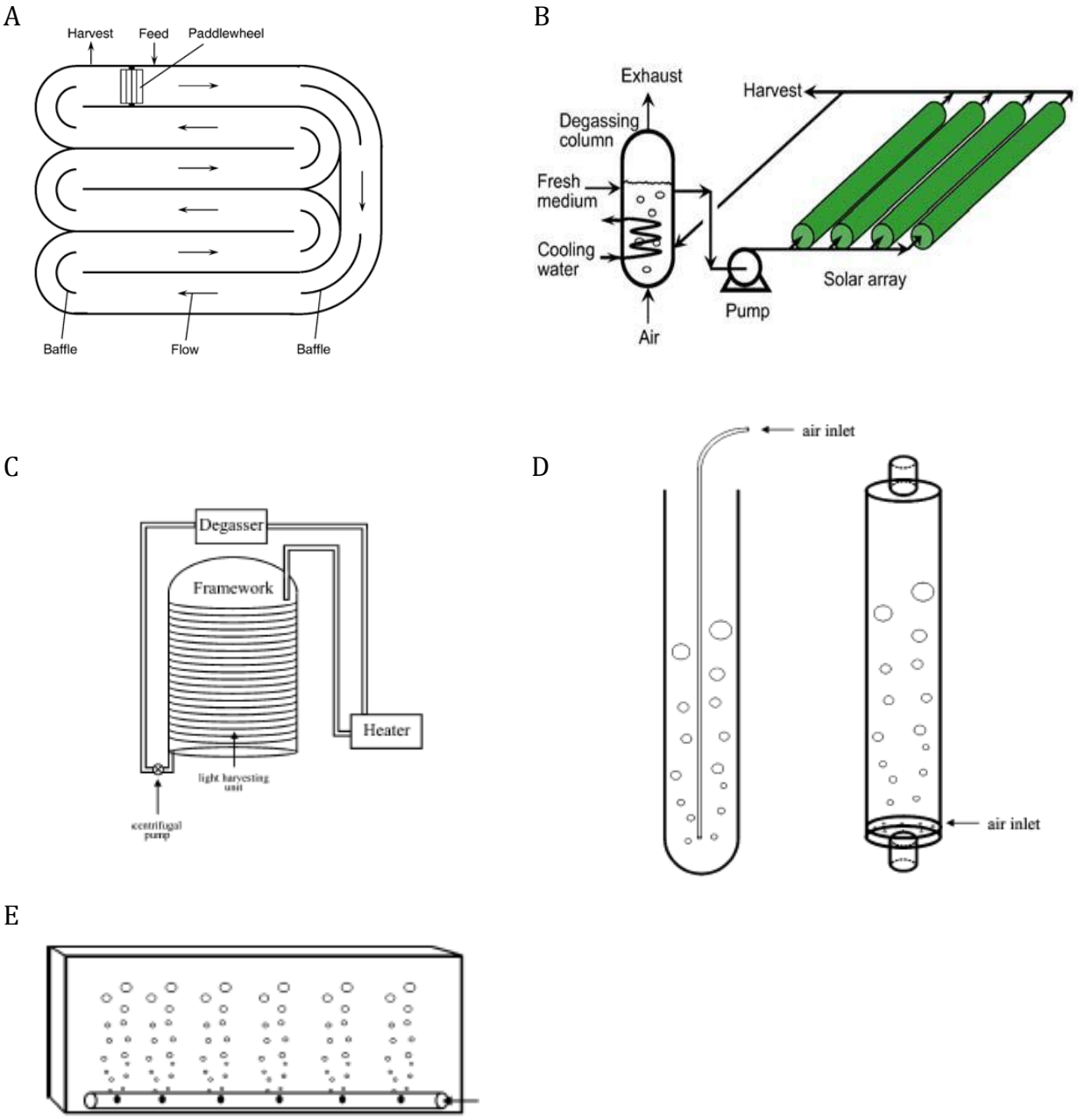


Fig. 2-1. Microalgae cultivation systems (open and closed systems). (A) Raceway pond [16]; (B) horizontal tubular [16]; (C) helical tubular [124]; (D) vertical-column [124] and (E) flat-plate photobioreactor [124].

Tubular photobioreactors are suitable for outdoor cultivation due to their large surface area for illumination. There are four types of tubular reactors: vertical, horizontal, helical and alpha-shaped. Alpha-shaped tubular reactors have high light efficiency whereas helical configurations require less space [124]. Many examples for applications of tubular reactors in microalgae cultivation were reported: vertical tubular reactor [125, 126], horizontal [127, 128], helical [129, 130] and alpha-shaped [129]. The second

design is the flat plate that promotes high productivity and uniform light distribution [124]. This type is also very suitable for outdoor cultivation. Some studies were carried out on this type [131, 132]. The last type of reactor is a vertical-column photobioreactor, which has poor light efficiency but, on the other hand, is excellent for controlling processing parameters and offers an alternative production process in the biochemical industry [124]. The various uses of vertical-column photobioreactors in microalgae cultivation have been reported [133, 134].

2.3. Harvesting of microalgae

Microalgae harvesting could be done using several techniques. Selection of the best harvesting process depends on size, biomass concentration and value of the product. A number of techniques for harvesting microalgae were investigated such as centrifugation [135], filtration [136], flotation [137, 138], chemical flocculation [139, 140], biological flocculation [141, 142], electroflocculation [143] and ultrasound [144]. Chemical and biological flocculations have low operating costs, but also some disadvantages such as being time consuming and a high risk of decomposition of the desired products. Other techniques (e.g., filtration, flocculation, centrifugation, flotation and ultrasound) are more efficient and safe for products, but more costly to operate.

A drying step is normally needed after harvesting; the correct choice of drying technique is important because it could influence the quality of the product. There are several techniques for drying including sun drying [145], spray drying [146, 147], fluidized bed drying [147], freeze drying [148] and refractance window (special thin film technique) dehydration technology [149]. The simplest and cheapest method of drying is by air using the sun. However, this method is time consuming, requires space, and causes a loss of products or diminished product quality. Other techniques have high costs, but are safe for bioproducts. Based on this, it is necessary to choose a harvesting strategy and drying technique that will offer the best balance between energy input and avoid damaging target compounds.

CHAPTER 3

Microalgae Lipids

3.1. Lipids in microalgae

Lipid compounds such as fat, oil, wax, fat-soluble vitamins, triacylglycerols, phospholipids, co-enzymes (ubiquinone), pigments (carotenoids), etc., can be found in animal or plants. Lipids are formed from long-chain hydrocarbons and sometimes contain other functional groups of nitrogen, phosphorus, oxygen and sulfur. They are insoluble in water, but soluble in organic solvents such as hexane, chloroform, ether, etc. The complete overview of lipids, different lipid class and lipid glossary can be accessed online (www.cyberlipid.org or www.lipidlibrary.co.uk).

Table 3-1. Lipid content of some microalgae species

Microalgae species	Lipid content (% dry weight)	Ref.
Chlorella sp.	32.6-66.1	[150]
Dunaliella tertiolecta	16.7	[151]
Nannochloropsis sp.	13-62	[151, 152]
Nannochloropsis oculata	22.7-45.5	[153, 154]
Neochloris oleoabundance	29-37	[110, 155]
Chlorella protothecoides	59 (photo); 14.57 (auto)	[107, 156]
Monodus subterraneus	39.3	[157]
Scenedesmus sp LX1	20-55	[158]
Chlorella vulgaris	56.6	[159]

Like higher plants, microalgae produce both neutral and polar lipids. There is a wide range of bio-based lipid products that can be harvested from the microalgae biomass. Microalgae lipids offer great potential in terms of biotechnology applications such as food, food supplements, energy, cosmetics, and pharmaceuticals. Microalgae are not only a source of food and dye, but they also have nutraceutical and cosmeceutical properties. In functional food, the use of microalgae lipids has already been established at industrial scales. The quality and type of the lipid products depend on microalgae species, culture conditions, and recovery methods. The fatty acid profile and composition of some microalgae species is shown in Table 3-2. The main fatty acids in

storage lipids are saturated fatty acids (e.g., palmitic acid, C16:0; stearic acid, C18:0) and unsaturated fatty acids (e.g., oleic acid C18:1; linoleic acid, C18:2; linolenic acid C18:3). However, polyunsaturated fatty acids with 20 and 22 carbon atoms are also found; these are ARA, DHA and EPA associated either with triacylglycerols or phospholipids.

3.2. Lipid biosynthesis

Polar lipids are synthesized under convenient environmental conditions, which are enriched in chloroplast and the cell membrane system [160, 161]. But if the environmental conditions are not suited to cell growth, many microalgae tend to accumulate neutral lipids to form lipid droplets decentralized in the cytoplasm as energy storage. Biosynthesis, which involves acetyl CoA as a precursor, produces malonyl CoA (as a precursor for synthesis of fatty acids) and mevalonate pathways (for the formation of carotenoids, steroids and terpenoids). Biosynthesis pathways of fatty acids and triacylglycerols (TAG) are particularly lipid metabolisms in algae that have been poorly studied compared to higher plants, with the exception of *Clamydomonas reinhardtii*, which is used for studying lipid metabolism [162]. The basic pathways of fatty acids and TAG biosynthesis in algae are analogous to higher plants, based on the data of the homology sequence and some biochemical characteristics of a number of genes and/or enzymes isolated from algae and higher plants that are involved in lipid metabolism. Lipids are accumulated through two different pathways: de novo synthesis and ex novo accumulation. The first pathway is a synthesis process from non-fatty acid precursors such as carbohydrates, amino acids or short-chain organic acids; then they produce acetyl Co-A, which is a precursor for this pathway. The second pathway involves the uptake of fatty acids, oils and TAG from the culture medium and their accumulation in an unchanged or modified form within the cell [163]. The de novo synthesis of fatty acids occurs in the chloroplast of algae cells and the general pathway scheme is shown in Fig. 3-1. An acetyl CoA is a basic material for building the fatty acid chain; it enters the pathway as a substrate for acetyl CoA carboxylase (1) as well as a substrate for the initial condensation reaction (3). The second reaction is catalyzed by malonyl CoA:ACP transferase, which serves to transfer malonyl CoA to malonyl ACP. Malonyl ACP is the carbon donor for subsequent elongation reactions and this reaction obtains ß-ketoacyl ACP, which is catalyzed by ß-keto-ACP. After subsequent condensations, the ß-ketoacyl ACP product is reduced by ß-ketoacyl ACP reductase (4), dehydrated process by ß-hydroxyacyl ACP dehydrase (5) and reduced by enoyl ACP reductase. The results of the

biosynthesis are C16, C18 fatty acids or both. The long-chain fatty acids are synthesized using palmitate (C16:0) as precursor, the process involves two enzymes, elongase and desaturase. Then, the route of biosynthesis of long-chain fatty acids are described in Fig. 3-2.

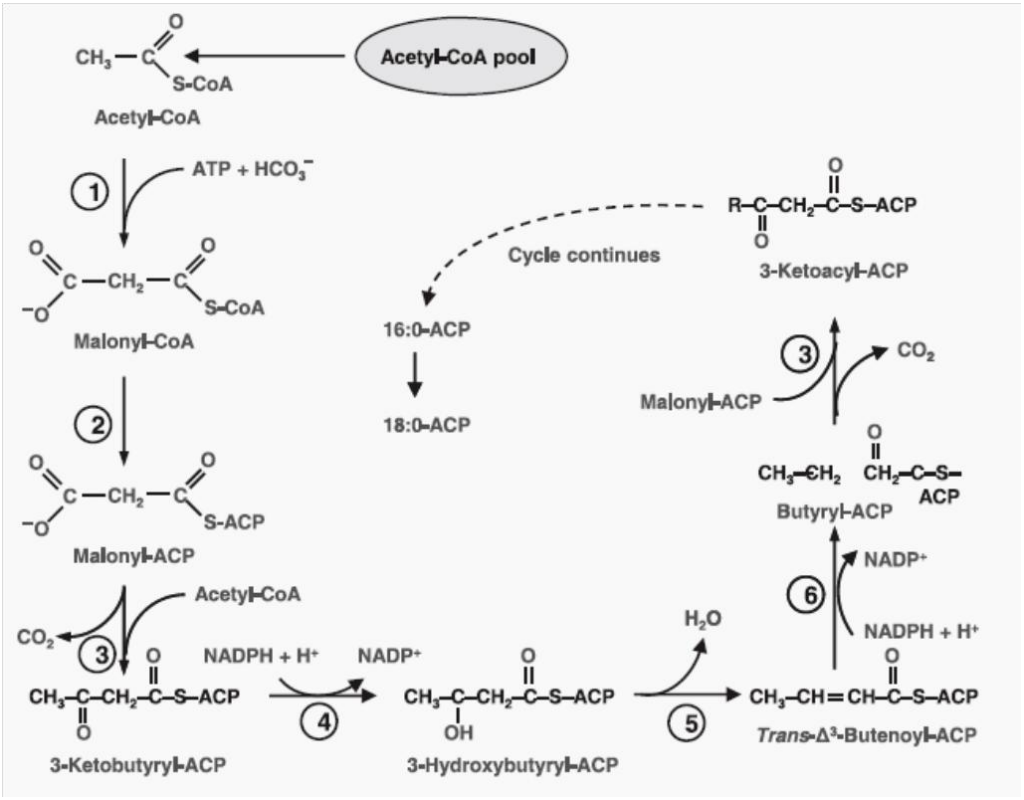


Fig. 3-1. Fatty acid synthesis pathway [164]. (1) Acetyl CoA Carboxylase (2) malonyl CoA:ACP (3) ß-keto-ACP synthase (4) ß-ketoacyl ACP reductase, (5) ß-hydroxyacyl ACP dehydrase and (6) enoyl ACP reductase.

The fatty acids C16 and C18 are used as the precursor for the synthesis of chloroplast and other cellular membranes such as synthesis of TAG. The synthesis of TAG, which involves 4 enzymatic steps involving 3 acyltransferases and a phosphatase, is called the Kennedy pathway. In algae, TAG biosynthesis has been suggested to occur via the direct glycerol pathway (Fig. 3-3); the results of biosynthesis fatty acids are transferred from CoA to positions 1 and 2 of glycerol-3-phosphate (G-3-P), which produces phosphatidic acid (PA). Dephosphorylation of PA is catalyzed by phosphatidic acid phosphatase which releases diacylglycerol (DAG). In the last step, a third fatty acid is transferred to the vacant position 3 of DAG and catalyzed by diacylglycerol acyltransferase. PA and DAG can also be used as a substrate for synthesis of polar lipids, such as phosphatidylcholine (PC) and galactolipids [165].

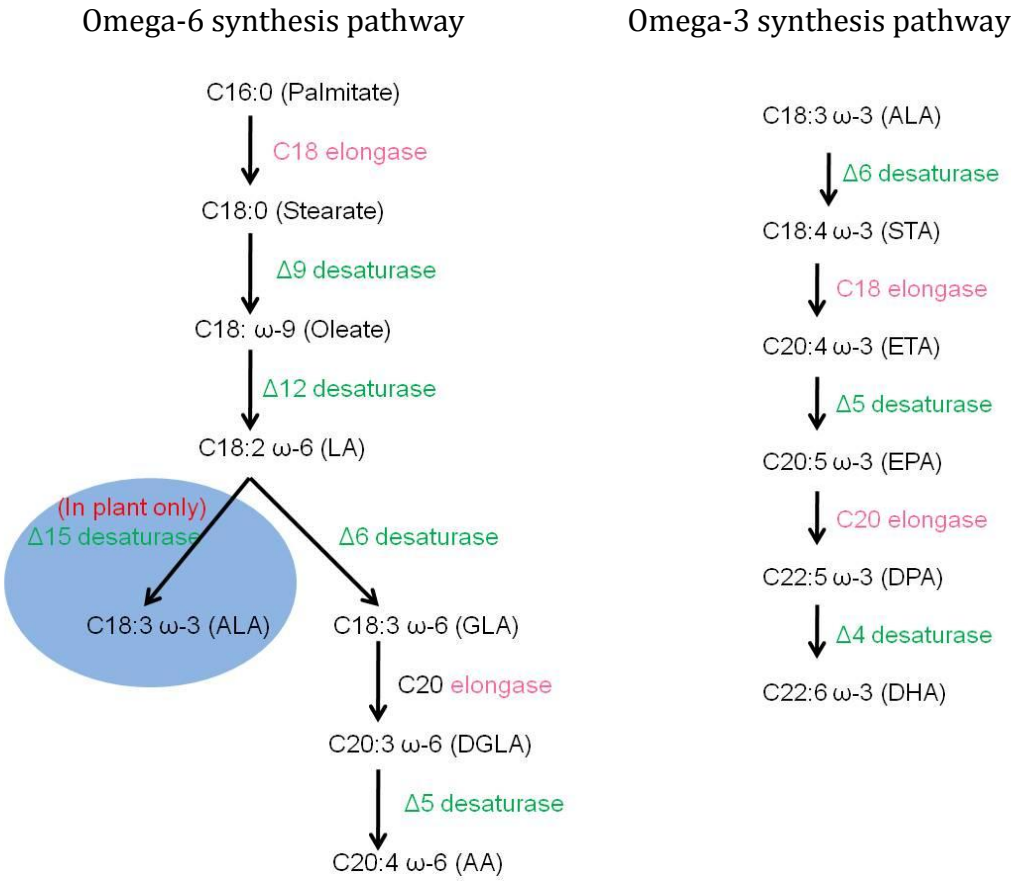


Fig. 3-2. Biosynthesis pathway of ω-6 and ω-3 fatty acids [166]. a) Omega-6 fatty acids include GLA (gamma-linolenic acid), DGLA (dihomo-gamma-linolenic acid) and AA (arachidonic acid) and b) Omega-3 fatty acids include ALA (alpha-linolenic acid), STA (stearidonic acid), ETA (eicosatetraenoic acid), EPA (eicosapentaenoic acid), DPA (docosapentaenoic acid) and DHA (docosahexaenoic acid).

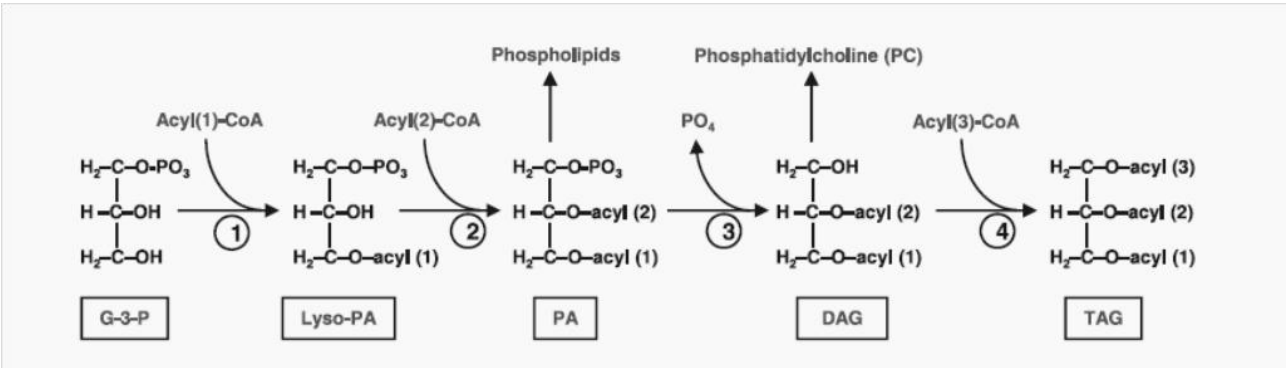


Fig. 3-3. The triacylglycerol biosynthesis pathway [165]. (1) Cytosolic glycerol-3-phosphate acyltransferase, (2) lyso-phosphatidic acid acyltransferase, (3) phosphatidic acid phosphatase and (4) diacylglycerol acyltransferase.

Table 3-2. The fatty acid profile in some microalgae species. Data are given as % total fatty acid.

Species	12:0	14:0	16:0	16:1	16:2	16:3	16:4	18:0	18:1	18:2	18:3	18:4	20:4	20:5	22:6	Ref.
Spirulina platensis			36.38	3.39				2.76	20.92	8.69	13.65					[167]
Scenedesmus sp.		0.27	29.52	0.76	0.52		1.96	2.33	17.83	7.99	18.50	3.29		0.84	2.11	[168]
Chlorella minutissima			25.97	2.20	12.47				21.03	11.00	27.33					[169]
Botryococcus braunii			29.50	3.40				1.00	44.90	21.20						[99]
Chlorella vulgaris			24.00	2.10				1.30	24.80	47.80						[99]
Phaeodactylum tricornutum		7.40	17.70	17.90	4.00	6.00	1.00	0.70	3.30	2.40	0.70	0.60	3.0	23.00	2.70	[170]
Chlorococcum humicola	1.06	0.64	41.78	0.97				2.53	20.37	14.43	15.81					[171]
Nannochloropsis sp.		4.50	25.30	23.40				0.90	5.20	2.20			6.3	30.80		[17]
Dunaliella tertiolecta			28.10		2.80	1.37		0.60	19.30	14.67	33.20					[172]
Tetraselmis sp.		0.60	27.80					0.90	28.20	9.30	23.90	3.70	0.9	3.40		[17]
Nannochloropsis salina		7.60	31.26	32.12					1.10	0.81	0.93		2.78	17.39		[118]
Isochrysis sp.		13.7	13.40	5.10				0.20	13.90	4.50	8.30	24.70	0.2	0.90	13.00	[17]
Nannochloropsis sp.	1.04	6.56	25.21	22.41					3.12	3.68	0.77		3.96	30.19		[173]
Porphyridium cruentum			34.10	1.10				0.90	1.60	11.50	0.50		40.30	6.60		[174]
Crypthecodinium cohnii	1.58	14.04	19.81					12.82	0.81						51.12	[175]

3.3. Factors affecting lipid synthesis

The growth of cells and lipid accumulation by microalgae under phototrophic conditions is influenced by nutrients, light and the environment. Several strategies have been developed to enhance microalgae lipid content under environmental stress. However, cultivation of microalgae under stress conditions results in low biomass yield with high lipid accumulation and hence overall low lipid productivity. The strategies include changing and limiting nutrient composition, introduction of optimal light management and variation of carbon dioxide or organic carbon sources (Table 3-3).

Table 3-3. Factors affecting lipid synthesis

Parameter	Lipid/TFA production	Species	Range	Ref.
Nitrogen (decrease)	↑	*Neochloris oleoabundans*	Stationary phase	[155]
		Chlorella sp.	Exponential-Stationary	[150]
		Nannochloropsis sp.	Stationary phase	[176]
		Nannochloropsis oculata	Exponential-Stationary	[154]
		Chlorella minutissima	Stationary phase	[169]
		Chlorella vulgaris	Stationary phase	[177]
Phosphorous (decrease)	↑	*Monodus subterraneus*	0 – 175 µM	[157]
		Nannochloropsis sp.	Stationary phase	[176]
Carbon:				
Acetate	↑	*Chlorella vulgaris*	1%	[178]
		Chlorococcum sp.	0 – 280 mM	[179]
Glucose	↑	*Chlorella protothecoides*	2 – 15 g L^{-1}	[107]
		Nannochloropsis sp.	2 g L^{-1}	[173]
Glycerol	↑	*Nannochloropsis* sp.	2 g L^{-1}	[173]
Sucrose	↑	*Nannochloropsis* sp.	2 g L^{-1}	[173]
Carbon dioxide		*Chlorella vulgaris*	0.33 – 3.33 %	[177]

	↑	*Nannochloropsis oculata*	0.04 – 15%	[153]
	↓	*Pavlova lutheri*	0.5 – 1%	[180]
Iron (increase)	↑	*Botryococcus* sp.	0 – 0.74 mM	[181]
		Chlorella vulgaris	0 – 0.012 mM	[159]
Salinity (increase, NaCl %)	↑	*Nannochloropsis* sp.	0.2 – 1.5 M	[152]
		Nannochloropsis sp.	13 – 40 g L^{-1}	[182]
		Botryococcus braunii	0 – 0.7 M	[183]
		Dunaliella	0.5 – 1 M	[184]
		Chlorococcum sp.	0 – 2% (w/w)	[179]
Light intensity (increase)	↑	*Nannochloropsis* sp.	17 – 700 µmol photons $m^{-2}.s^{-1}$	[182]
		Pavlova lutheri	9 – 30 W m^{-2}	[185]
		Pavlova lutheri	75 – 120 µmol photons $m^{-2}.s^{-1}$	[180]
		Pariethochloris incisa	35 – 400 µmol photons $m^{-2}.s^{-1}$	[186]
Light photoperiod (increase)	↑	*Chlorella minutissima*	LD: 24 – 0 h	[169]
		Chlorococcum sp.	LD: 24 – 0 h	[179]
	NA	*Nannochloropsis* sp.	LD: 24 – 0 h	[187]
Temperature (increase)	↑	*Scenedesmus* sp.	10 – 30°C	[120]
		Chaetoceros	25 – 35°C	[119]
	↓	*Nannochloropsis oculata*	15 – 25°C	[117]
pH (Increase)	NA	*Chlorella*	7-11	[160]

3.4. Lipid extraction

Today, large-scale lipid extraction technologies are available on the market that could be applied for microalgae oil and were reviewed by Mercer and Armenta [188] and Cooney et *al*. [189]. Table 3-4 describes the benefits and limitations of the extraction methods for recovering oil. Organic solvents, such as benzene, hexane, chloroform, etc., are a widely used method for the recovery of oil from microalgae biomass. The organic solvents used in industrial applications should be cheap, non-toxic, have a low boiling point or be easy to remove after extraction, insoluble in water, efficient in dissolving target compounds, and reusable. Hexane is an organic solvent which meets all the criteria for application in large-scale industrial processes. The methods of Bligh-Dyer [190] and Folch et *al*. [191] have been widely used for oil recovery from plants and animals. Both methods use chloroform, which is toxic and carcinogenic. Sanchez et *al*. [192] replaced chloroform with the less toxic dichloromethane. The extraction of microalgae oil with 96% ethanol was investigated by Fajardo et *al*. [170].

Super fluid extraction (SFE) is a relatively new technique in the oil extraction process. This method produces highly purified products without using harmful solvents and is not time consuming; however, it is expensive. The application of supercritical CO_2 in lipid extraction was reviewed in detail by Sahena et *al*. [193].

Ultrasound-assisted extraction (UAE) is an extraction process through acoustic cavitations and some mechanical effects, in which acoustic cavitations can disrupt cell walls facilitating the solvent to penetrate the plant material and allow intracellular release of the product. Ultrasound-assisted extraction can be carried out at a lower temperature which can avoid thermal damage to the extracts and minimize the loss of bioactive compounds [194, 195]. Some researchers investigated the use of this method for microalgae species [177, 196, 197].

Pulsed electric field (PEF) is a method for processing cells by means of brief pulses of a strong electric field; a substance is placed between two electrodes and then the pulsed electric field is applied. PEF treatment is used in algae extraction and could potentially provide a means of gentler downstream processing [198]. Koehler et *al*. [199] investigated the extractability of proteins, chlorophyll, and carotenoids as well as the protease activity of extracts from *Spirulina* and *Chlorella*, the extractability of growth hormone formulations from Kelp after a PEF treatment indicating an improved yield after pressing.

Others promising techniques are microwave-assisted extraction (MAE) and electroporation methods. MAE is a process that includes heating the material causing moisture to evaporate, which generates tremendous pressure on the cell wall causing the desired contents to be released from the cells. In this case heating occurs only in a selective and targeted material; consequently there is no heat release into the environment [200, 201]. MAE has been successfully demonstrated for extraction of microalgae lipid in *Scenedesmus obliquus*, with an oil recovery yield that is 30% more than with a water bath system [202].

An alternative method that is relatively safe and environmentally-friendly, is using enzymes to breakdown cell walls of microalgae to release cell contents. The use of enzymatic treatment has the potential to partially or fully disrupt cells with minimal damage to the product. On the other hand, this technique is expensive because the enzymes cannot be recovered and recycled after use [203]. Consequently, this method is not widely used in industry. Enzymatic treatment has been applied for degradation of cell walls in *Chlorella vulgaris* using cellulose and lipase enzymes [204].

Electroporation is a membrane phenomenon which involves a significant increase in the electrical conductivity and permeability of the cell wall and cytoplasm membrane resulting from an externally applied electrical field. This process results in the formation of aqueous pores in the membrane [205].

Table 3-4. Benefits and limitation of extraction methods for recovering oil from microalgae

Method	Benefits	Limitation	Ref.
Presses	Easy and no organic solvent, capable to extract up to 80% oil.	Slow process and requires large number of samples, high cost.	[206, 207]
SE	Required solvents are inexpensive, reproducible.	Most organic solvents are toxic and flammable, solvent recovery is expensive, large volume of solvent is required.	[58, 208-210]
SFE	Non-toxic solvent, non-flammable, simple operation, cheap, reduce solvent waste and environmentally friendly.	High energy consumption and difficult to scale up.	[211, 212]
UAE	Reduce extraction time and solvent consumption, greater penetration of solvent into cellular materials, improved release of cell contents into bulk medium.	High energy consumption and difficult to scale up in industry.	[194, 195, 213, 214]
PEF	Non-thermal process, short extraction time, reduces heating effects.	High power source requirement, scaling up is difficult, safety issue from bubble formation leading to electrical breakdown of the treated product.	[198, 199]
MAE	Quick, safe and inexpensive method, reduces extraction time and does not require initial dehydration of biomass.	Oxidative damage to valuable lipid products and difficult to scale up.	[200, 202, 214, 215]
Enzymatic	Safe and environmentally friendly, minimal damage to the product.	Requires designing effective enzyme, the enzyme is not commercially available, time consuming and expensive.	[204]
Electroporation	Reduced extraction time and quantity of solvents, does not cause significant changes in the composition or oxidation of products.	Required synthesis of membrane, depend on the material dielectric properties.	[205]

SE: solvent extraction, SFE: super fluid extraction, UAE: ultrasound-assisted extraction, PEF: pulsed electric field, MAE: microwave assisted-extraction.

Table 3-5. Works published on the recovery and analytical technique of microalgae lipid

Functional groups	Microalgae species	Extraction method	Analytical technique	Ref.
Carotenoids:				
Astaxanthin	*Haematococcus pluvialis*	SFE	HPLC, LC- (UV/Vis)	[216]
	Chlorococcum sp.	SE	HPLC,HSCCC	[217]
β-carotene	*Dunaliella salina*	SFE	HPLC, LC- (UV/Vis)	[218]
Lutein, violaxanthin, neoxanthin	*Phormidium*	PLE	HPLC-DAD	[219]
Nostoxanthin	*Nostoc commune*	Ultrasounds	HPLC, FD-MS	[220]
Canthaxanthin	*Chlorella emersonii*	SE	HPLC	[221]
DHA	*Crypthecodinium cohnii*	SFE	GC, GC-MS	[222, 223]
	Aurantiochytrium sp.			[224]
EPA	*Phaedactylum tricornutum*	SE	GC	[170]
	Nannochloropsis sp.		GC	[128]
ARA	*Nannochloropsis* sp.	$ScCO_2$	GLC	[211]
GLA	*Spirulina platensis*	SFE	GC-FID	[225]
		PLE	HPLC/ESI-QTOF-MS	[226]
	Spirulina maxima	SFE, SE	GC	[227]
ALA	*Nannochloropsis* sp.	$ScCO_2$	GLC	[211]
Oleic acid	*Spirulina platensis*	SFE	GC-FID	[228]
Palmitoleic acid	*Spirulina platensis*	SFE	GC-FID	[228]
Vitamin E	*Porphyridium cruentum*	Ultrasounds	HPLC-FD	[47]
	Spirulina platensis	SFE	HPLC-FD	[228]
Glycerolipid	*Chaetoceros muelleri*	SFE	HPLC-ELSD, HPLC-QTOF-MS	[61]
Glycolipid	*Spirulina platensis*	SE	HPLC	[229]

SFE, Super Fluid Extraction; SE, Solvent Extraction; QTOF, Quadrupole-Time of Flight mass analyzer.; Mass Spectrometry (MS); High-performance Liquid Chromatography (HPLC); Ultraviolet Detector (UV); Flame Ionization Detector (FID): Fluorescence Detector (FD); Pressurized liquid extraction (PLE), Diodenarray detector (DAD), Electrospray ionization (ESI); High-speed counter-current chromatography (HSCCC).

3.5. Lipid analysis

Traditional total lipid analysis is a macro-gravimetric method; lipids are extracted from matter, the solvent is evaporated and the retained material is measured as the lipid content [190, 191]. Macro-gravimetric requires a relatively large quantity of sample and is time consuming when the analysis of many samples is needed. A colorimetric sulfo-phospho-vanillin (SPV) method was developed for lipids analysis. The method uses a reaction mixture (sulfuric acid, phosphoric acid and vanillin) maintained in a 96-well microplate. The method has been used for determination of total lipids in serum, plasma, food, and microalgae biomass [230, 231]. According to several investigators, the SPV method allows for rapid processing and is efficient in terms of solvent usage compared to gravimetric methods [232, 233].

Spectrofluorometry uses the fluorescent dye Nile red that was originally developed by Greenspan et *al*. [234] and has been modified for quantification of total lipids in microalgae species [235, 236]. Proteins and pigments will cause interference and the fluorescence intensity varies between samples [237]. Therefore, an accurate quantification of lipids using this method requires determination of the optimal excitation and emission wavelengths for maximum fluorescence of each type of sample prior to fluorescent measurements [235, 238]. For example, green algae species which contain chlorophyll (1-4% of dry matter) will increase the fluorescence background; this will make it difficult to quantify lipids using Nile red. This is a reason why the optimal excitation and emission wavelengths must be identified [235]. The 96-well-plate-based Nile red method can be used as a high-throughput technique for rapid screening.

Laurens and Wolfrum [239] investigated the tandem of near infrared (NIR) and Fourier transform infrared (FTIR) spectroscopy for predicting the levels of spiked neutral and polar lipids in microalgae. The advantage of this method is direct, fast, and non-destructive nature of the screening method. Beal et *al*. [240] also evaluated the use of liquid state ^{13}CNMR for analyzing the lipid composition of *Neochloris oleoabundans* for biodiesel production. According to them, NMR is a useful analytical tool for selectively identifying algae lipid, especially for biodiesel production.

Analysis of fatty acids (FA) is done in several steps involving the extraction of lipid from the sample, the isolation of free fatty acids from the rest of the lipids; derivatization of FA to fatty acid methyl ester (FAME); and analysis of FAME by gas chromatography (GC), gas chromatography mass spectrophotometer (GC-MS) or high-

performance liquid chromatography (HPLC) [241]. Another method for the analysis of FA is via the direct transesterification method; this method has been applied to microalgae, cells, tissue, and infant formulae [242, 243]. The samples are reacted with methylation reagent for the derivatization of FA to FAME. This method provides a simple analytical procedure for the analysis of long-chain polyunsaturated fatty acids [242]. The detailed FA analysis was reviewed by Rodrigues et *al*. [241] and Bernal et *al*. [244], including sample preparation and analysis with different equipment. Table 3-5 describes the common methods of extraction and analytical techniques of lipid products from microalgae.

Based on the description above, the analysis of fatty acids usually uses expensive analytical instruments (e.g., GC, GC-MS or HPLC), which are not always available in many laboratories, but there is a simple and fast method for quantification of fatty acids from algae lipids using colorimetry [245]. This technique was derived from a method for the quantification of fatty acids dissolved in chloroform [246]. According to Wawrik and Harriman [245], the detection and quantification of fatty acids with this method is simple, fast and only uses a small amount of culture (1-2 ml of culture); it is also cost saving as analysis costs less than $5 per sample.

CHAPTER 4
Materials and General Methods

4.1. Chemicals

All media components and solvents used were purchased from either Merck (Germany), Sigma-Aldrich (USA) or Roth (Germany). All chemicals used in the experiments were ACS grade. Crude glycerol K501 was provided by Biopetrol Schwarzheide GmbH (Germany). The analysis provided by the manufacturer stated the composition contained 48% (w/w) glycerol, 44% (w/w) water, 0.00096% (w/w) chloride, and 3.4% (w/w) ash.

4.2. Microalgae and media

Chlorococcum sp. and *Nannochloropsis* sp. were obtained from culture collection by the Research Center for Biotechnology, Indonesia Institute of Science (LIPI), Indonesia. Tables 4-1 and 4-2 describe the composition of BG-11 [247] and f/2 medium [248].

Table 4-1. The composition of BG-11 and f/2 medium

Component	Stock solution ($g\ L^{-1}$)	BG-11 medium (quantity per 1 L of water, mL)	F/2 medium (quantity per 1 L of artificial sea water, mL)
$NaNO_3$	150	10	1
K_2HPO_4	30	1	-
$MgSO_4.7H_2O$	75	1	-
$CaCl_2.2H_2O$	36	1	-
Citric acid	6	1	-
Ferric Ammonium Citrate	6	1	-
EDTA	1	1	-
Na_2CO_3	20	1	-
$NaH_2PO_4.H_20$	5	-	1
Vitamin solution	See below	-	0.5
Trace Metal solution	See below	1	0.5

Chlorococcum sp. was maintained in BG-11 medium at pH 7.5 and f/2 medium in artificial sea water (32%, Tropic Marin®) was used for *Nannochloropsis* sp. Inoculates for all experiments were added from a 10-day-old culture without aeration and grown under continuous illumination at 27 µmol photons m^{-2} s^{-1} at 22 ± 2°C with agitation at 100 rpm.

Table 4-2. Trace metal solution and vitamin solution

Component	**Stock solution (g L^{-1})**	**Quantity per 1 L H_2O**
Trace metal solution BG-11 medium		
H_3BO_3	2.86	-
$MnCl_2.4H_2O$	1.81	-
$ZnSO_4.7H_2O$	0.222	-
$Na_2MoO_4.2H_2O$	0.390	-
$CuSO4.5H_2O$	0.079	-
$Co(NO_3)_2.6H_2O$	0.0494	-
Trace metal solution for F/2 medium		
$FeCl_3.6H_2O$	-	3.15 g
$Na_2EDTA.2H_2O$	-	4.36
$CuSO_4.5H_2O$	9.8	1 mL
$Na_2MoO4.2H_2O$	6.3	1 mL
$ZnSO_4.7H_2O$	22	1 mL
$CoCl2.6H_2O$	10	1 mL
$MnCl.4H_2O$	180	1 mL
Vitamin solution		
Thiamine HCl		200 mg
Biotin	1	1 mL
Vitamin B12	1	1 mL

4.3. Experimental set up

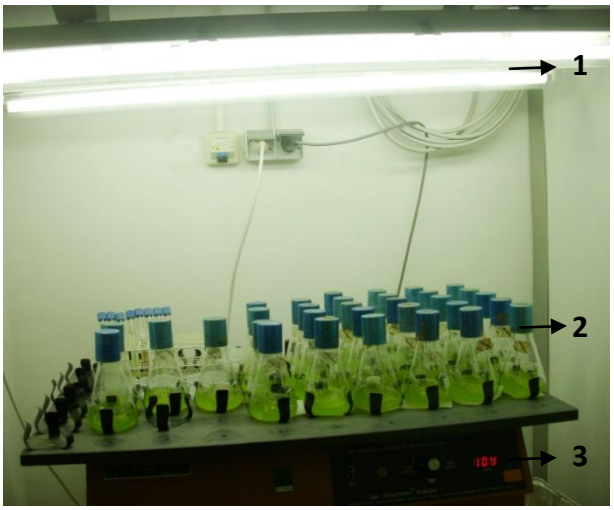


Fig. 4-1. The experimental set up in flask 250 mL.

(1) TL Lamp

(2) Flask

(3) Shaker

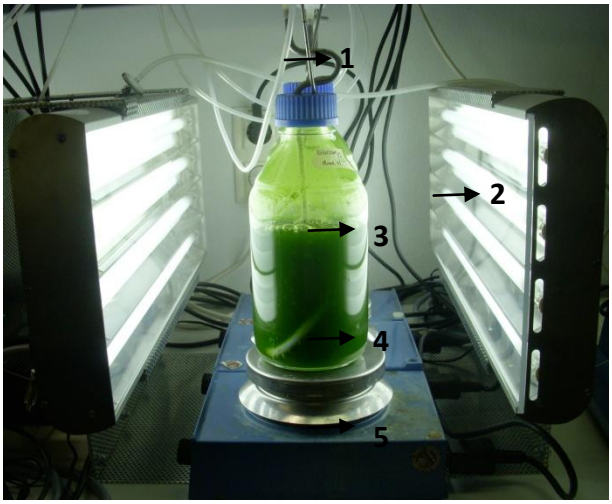


Fig. 4-2. The experimental set up in flask 1-L.

(1) Silicon tube (air/CO_2 gas)

(2) TL Lamp

(3) Flask

(4) Flexible tube pore

(5) Magnetic stirrer

4.4. Growth measurement, cell staining and dry weight determination

Cell growth was monitored daily by optical density at 680 nm with a spectrophotometer (T80 UV/VIS Spectrometer) and cell number was counted with an optical microscope (Zeiss Axioplan) using a haemacytometer (Thoma). One unit of OD_{680} corresponded to 10^7cells/ml. Cellular lipid droplets were stained with Nile Red solution, briefly Nile Red solution (250 µg mL^{-1} in acetone) and acetone were added to cells in the growth medium and the final concentrations were 1 µg mL^{-1} and 0.04% (v/v), respectively [249]. After 30 s, the stained cells were observed under a fluorescent microscope. Biomass was harvested by centrifugation at 4500 rpm for 7 min and the

cells were rinsed twice with distilled water. The washed biomass was oven dried at 80°C for 24 h; the dry biomass was weighed and pulverized to a fine powder using a mortar.

4.5. Measurement of nitrate, acetate, glycerol, phosphate and carbon dioxide

The inorganic nitrogen content was measured using ion chromatography. Acetate content was quantified using high-performance liquid chromatography (HPLC-UV Shimadzu). Carbon dioxide was monitored with a carbon dioxide sensor (BlueSens Gas Sensor).

4.5.1. Measurement of nitrate

The IC-HPLC system configuration consisted of a UV Detector at 210 nm and a column C18 ODS Hypersil (125 x 4.0 mm; 5µm; 120A). The chromatography was performed at room temperature with a mobile phase composed of 7.5% (v/v) methanol, 0.2% (v/v) octylamin and deionized water. The solution was adjusted to pH 6.5 with phosphoric acid. The following elution program was used: 20 µL injection volume, 2 mL min^{-1} flow rate and 3 min operational time. A nitrate concentration in the range 0–100 ppm was used as standard.

4.5.2. Measurement of phosphate

The phosphate was measured using a spectrophotometer according to Mahadevaiah et *al.* [250]. A series of volumetric flasks were arranged, and into each flask was added 0.5 mL of 0.387 M ammonium molybdate, 3 mL of 0.25 N sulfuric acid and aliquots of disodium hydrogen phosphate corresponding to 0.3-12.24 ppm (0.1, .2, 0.3, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 mL, with working solution 0.0458 g L^{-1}). Sodium sulphide (1 mL) of 2.0833 x 10^{-3} M solution was added to each flask. The solutions were reacted at room temperature for about 20 min. The absorbance was measured at 715 nm against water.

4.5.3. Measurement of acetate and glycerol

The IC-HPLC system configuration included the following: HPLC pump (Knauer K-1001); RI detector (Knauer K-2301), UV-Detector at 210 nm; column Aminex® HPX-87H 300 x 7.8 mm. A mobile phase was composed of 5 mM H_2SO_4 in deionized water. The following elution program was used: 50 µL injection volume, 0.7 mL min^{-1} flow rate, 60°C column temperature, 35°C detector temperature and 12 minutes run time. Acetate and glycerol concentrations in the range of 0 – 2 g L^{-1} were used as standard.

4.6. Lipid analysis

4.6.1. Total lipid extraction

Total lipid content was extracted from dry mass using dichloromethane-methanol (2:1, v/v) [251]. Briefly, 100 mg of biomass powder and 3 mL dichloromethane-methanol (2:1, v/v) were mixed and stirred for 2 h. After the process was completed, the samples were separated and transferred to a new tube. The residue was then extracted for the second time in dichloromethane-methanol (2:1, v/v). Sample solutions were collected and added to 1.25 mL of KCl solution (0.88%, w/v) and stirred vigorously. The addition of KCl solution led to phase separation; the upper phase was discharged and the bottom phase was evaporated under nitrogen gas. Total lipid contents were analyzed gravimetrically. The extraction was carried out in duplicate.

4.6.2. Fatty acid analysis

The fatty acids were determined by the direct transesterification of algae biomass [252]. Briefly, 20 mg of biomass and 3 mL of transesterification reaction mix (methanol/ hydrochloric acid/ chloroform, 10:1:1 vol/vol) were added. Cells were vortexed for 10 s and were placed at 90°C for 120 minutes. After the process was completed, the samples were removed and allowed to cool down to room temperature. Water (1 mL) was then added and vortexed for 10 s. The fatty acid methyl esters (FAME) were extracted via the addition of 3×2-mL aliquots of hexane, vortexed and separated. The FAME were analyzed using gas chromatography equipped with a flame ionization detector (GC 17A-FID, Shimadzu) using an INNOWAX capillary column (60 m length, 0.25 mm inner diameter, 0.25 µm film thickness) and an autosampler. The injection port and detector temperature were 250°C and 300°C, with a split ratio (50:1), using 2.25 ml/min hydrogen as carrier gas and 1 µl injection volume. The oven temperature increased from 60°C to 150°C at 30°C/minutes, followed by an increase to 240 ^{o}C at a rate of 13°C/min and a hold at 240°C for 30 minutes. The temperature was then increased to 265°C and finally held for 5 minutes. GC analysis of FAME was carried out using an internal standard (5 mg methyl nonadecanoate, Sigma), which was added directly before analysis. FAME peaks were identified and calibrated with the corresponding standard fatty acid methyl esters.

CHAPTER 5

Lipid Production of Freshwater Microalga *Chlorococcum* sp.

5.1. Introduction

Chlorococcum is of the family Chlorococcaceae, which belongs to single-celled green microalgae. *Chlorococcum* is found in both aquatic and terrestrial habitats. Cells are spherical or slightly oblong in shape and variable in size [1]. This group of algae is non-motile, but some taxa produce motile cells (planospores) in the vegetative state [253]. Fig. 5-1 shows cells of *Chlorococcum* sp.

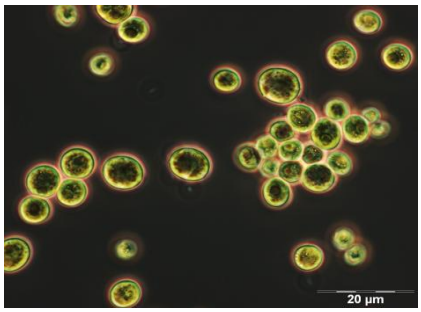


Fig. 5-1. Cells of *Chlorococcum* sp. under light microscope

Microalgae can be cultivated under photoautotrophic and mixotrophic conditions. It is well known that cultivation under photoautotrophic conditions has several disadvantages including low cell density and long cultivation time. Heterotrophic and mixotrophic cultivations have been proposed as potential alternatives for the production of microalgae biomass [173, 254] and to enhance lipid content in the microalgae cells [107, 255, 256]. Heterotrophic cultivation uses an organic compound as the sole carbon source, while mixotrophic cultivation applies inorganic and organic compounds as the carbon source [257]. Cultivation under mixotrophic conditions offers several advantages including high production rate, lower energy cost, and higher biomass and lipid productivity than cultivation under photoautotrophic conditions [178, 258, 259]. Various sources of carbon can be used to make microalgae production low cost. Some papers reported that crude glycerol from biodiesel production, acetate from anaerobic digestion, and carbohydrates from agriculture and industrial waste offer great potential as inexpensive organic substrates for microalgae cultivation under mixotrophic conditions [260, 261].

5.2. Methods

5.2.1. Tolerance to pH, salt and carbon dioxide

Chlorococcum sp. was grown at an initial pH from 6.5 to 9.0 to determine the optimum pH. Halo tolerance was determined using concentrations of 0 to 2% (w/v) sodium chloride. The cultures were aerated with air containing 1, 3, 6 and 10% (v/v) CO_2, respectively, to determine CO_2 utilization, biomass concentration and total lipid content. Cells were cultivated at 28 $\pm$ 2°C under continuous illumination at 54 µmol photons m^{-2} s^{-1} for 10 days of cultivation.

5.2.2. Effect of nitrogen source, yeast extract concentration and nitrate concentration

Nitrogen source including KNO_3, $NaNO_3$, NH_4NO_3 and yeast extract were used in cell cultivation. Cells were grown in different nitrogen sources with a concentration of 1.5 g L^{-1} for 10 days of cultivation. Different concentrations of yeast extract were also investigated regarding growth and lipid content. Cells of *Chlorococcum* sp. were inoculated in BG-11 medium with different concentrations of yeast extract (0.1 – 6 g L^{-1}). Cells were also inoculated in the BG-11 medium with different nitrate (from $NaNO_3$) concentrations (0.2, 1.2, 8.8, 17.7, and 35.3 mM) to determine the effects of nitrate concentration on growth and lipid content. BG-11 medium containing nitrate 1.2 mM is named modified BG-11 medium. Cells were cultivated at 28 $\pm$ 2°C under continuous illumination at 54 µmol photons m^{-2} s^{-1} for 10 days of cultivation.

5.2.3. Effect of glucose and acetate as carbon substrate and concentration of inoculum

Cells were inoculated in the BG-11 medium supplemented with acetate (1 g L^{-1}) and glucose (1 g L^{-1}) for 10 days of cultivation. The concentrations of acetate (0 – 280 mM) were used to determine the effects of acetate concentration on growth and lipid content. The responses of growth and total lipid content in different inoculums of algae cells were also evaluated.

5.2.4. Effect of light dark cycles on biomass total lipid and fatty acid content

Cells were grown in the modified BG-11 medium and supplemented with 70 mM acetate or without acetate in a 2-L flask. Inoculums were added from 1-L of a 7-day-old culture and grown under continuous light at 90 µmol photons m^{-2} s^{-1}. Cultures were centrifuged, washed twice with deionized water and cultivated in 1800 ml medium. The cultures were agitated at 100 rpm, at 28 $\pm$ 2°C with a light illumination of 90 µmol

photons m^{-2} s^{-1} on a LD 12:12 h and 24:0 h for 14 days. The cultures were cultivated at constant aeration under filtered air flow.

5.2.5. Effect of light intensity on biomass, lipid and fatty acid content

Cells were grown in the modified BG-11 medium in a 1-L flask and supplemented with 70 mM acetate or without acetate and in the modified BG-11 medium and supplemented with 70 mM acetate and sodium chloride 1% (w/v). Cells were cultivated under different light intensities to determine the effects of light utilization on growth, total lipid content and total fatty acid content. Light intensity was 100, 250 and 500 µmol photons $m^{-2}s^{-1}$ provided by a white fluorescent lamp on an LD 12:12 h.

5.2.6. Effect of different types of salt on biomass, lipid and fatty acid content

Cells were grown in the modified BG-11 medium of nitrate and supplemented with 70 mM acetate or without acetate, supplemented with sodium chloride 1% (w/v) and potassium chloride 1% (w/v). Cells were cultivated under light intensity of 250 µmol photons m^{-2} s^{-1} provided by a white fluorescent lamp on a LD 12:12 h.

5.2.7. Two-stage cultivation

Cells were grown in the BG-11 medium in a 1-L flask containing 1.5 g L^{-1} sodium nitrate for 7 days. After first cultivation, the cells were centrifuged and washed with deionized water and then grown in different media. In the second stage, the cells were grown in the BG-11 medium without sodium nitrate and containing carbon source including acetate, glucose, glycerol and crude glycerol for 7 days. Afterwards, the cells were cultivated under light intensity of 250 µmol photons m^{-2} s^{-1} provided by a white fluorescent lamp on a LD 12:12 h.

5.3. Results and discussion

5.3.1. Tolerance to pH, salt and carbon dioxide

A pH value is one of the environmental factors determining the growth of microalgae cells. The cultures of *Chlorococcum* sp. were able to grow in a wide pH range from 6.5 to 9.0 under photoautotrophic conditions. However, the optimum growth was between pH 8.0 and 8.5 (Fig. 5-2.). Microalgae can grow in brackish water or seawater, salinity is a factor determining biomass concentration and lipid accumulation in algae cells. In this study, effects on growth and lipid accumulation of *Chlorococcum* sp. were observed (Table 5-1). The salinity effect was observed with concentration from 0% - 2%

(w/v) of NaCl; cells can be grown in up to 2% (w/v) NaCl. In contrast to total lipid content, biomass growth decreased with salinity increase. The total lipid content was obtained in the medium containing 2% (w/v) NaCl, but the optimum lipid productivity was reached in the medium containing 1% (w/v) sodium chloride.

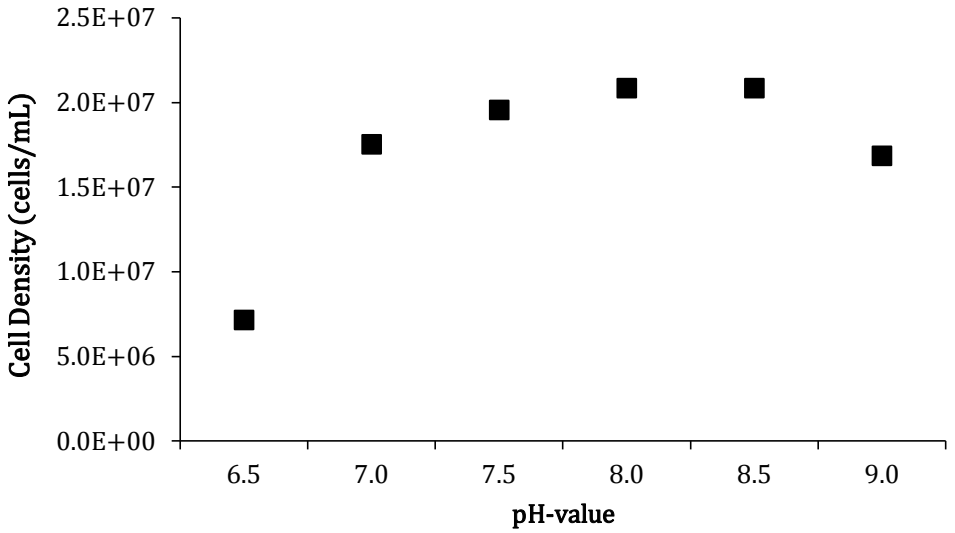


Fig. 5-2. Growth of *Chlorococcum* sp. at different pH in BG-11 medium.

Results indicated that salinity was one method to improve the total lipid content of *Chlorococcum* sp. Salinity is reported to be a factor in increasing the lipid content of algae; increases in salinity may result in a slight increase in total lipid production [262]. These results are in agreement with other researchers who reported an increase in the total lipid content of *Dunaliella tertiolecta* from 60% of dry weight with an initial NaCl concentration of 0.5 M, to 67% of dry weight with an initial NaCl concentration of 1.0 M [184]. Cell sizes were 4.9–13.4 μm in a BG-11 medium containing sodium chloride.

Table 5-1. Biomass, lipid productivity and total lipid content of *Chlorococcum* sp. in different salt concentrations.

Concentration of NaCl (%)	Biomass (dry weight, g L^{-1})	Lipid productivity (mg L^{-1} d^{-1})	Total lipid content (% of dry weight)
0	0.60	6.2 ± 1.7	10.3 ± 2.9
0.5	0.65	10.0 ± 1.0	15.4 ± 2.8
1	0.64	11.3 ± 1.2	17.7 ± 3.3
2	0.14	4.0 ± 0.2	29.8 ± 2.0

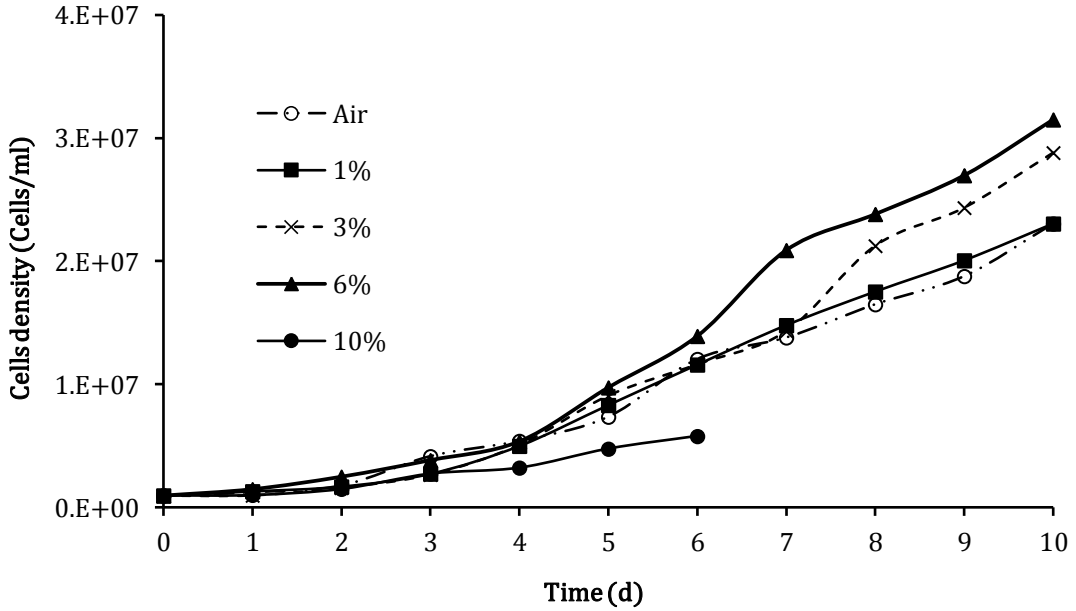


Fig. 5-3. Growth curves of *Chlorococcum* sp. in the BG11 medium under different levels of CO_2. Concentrations of CO_2 applied were ○, Air; ■, 1% (v/v); ×, 3% (v/v); ▲, 6% (v/v); and ●, 10% (v/v).

Carbon dioxide is an important factor in microalgae growth and lipid biosynthesis. Most microalgae grow well under concentrations of CO_2 from 1% to 5% (v/v). A concentration of CO_2 above 5% (v/v) could be harmful to microalgae cells and inhibit the growth of microalgae [153], but there are some species that are able to grow at high CO_2 concentrations [263]. A number of studies reported that CO_2 concentration has an effect on biosynthesis of lipid using several species of microalgae and concluded that CO_2 seems to control lipid content [153, 177]. The effect of CO_2 concentration on both growth and lipid content in *Chlorococcum* sp. was observed. The cultures were aerated under different levels of CO_2 with the same flow rate. Fig 5-3 shows the effect of CO_2 concentration on growth. The results show that *Chlorococcum* sp. was able to use up to 10% (v/v) carbon dioxide. The highest concentration of biomass was obtained in the medium at a CO_2 concentration of 6% (v/v) and reached the maximum biomass of 1.32 g L^{-1} after 10 days of cultivation. On the other hand, low biomass concentration and total lipid content were achieved when the cultures were aerated with 10% (v/v) of CO_2 (Table 5-2). It could be concluded that aeration of CO_2 with a concentration above 6% (v/v) was harmful for *Chlorococcum* sp. An increasing concentration of carbon dioxide from one to six percent CO_2 enhanced growth and total lipid content, and these results agree with the data reported by Widjaja et *al.* [177]

Table 5-2. Biomass, lipid productivity and total lipid content of *Chlorococcum* sp. at different levels of CO_2.

CO_2 concentration (%, v/v)	Biomass (dry weight, g L^{-1})	Lipid productivity (mg $L^{-1}d^{-1}$)	Total lipid content (% of dry weight)
0.04	0.53	5.3 ± 1	10.3 ± 2.9
1	1.04	16.8 ± 2	16.2 ± 3.5
3	1.03	14.9 ± 2	14.5 ± 1.1
6	1.32	19.3 ± 1	14.6 ± 2.8
10[a]	0.23	2.0 ± 0.2	6.1 ± 2.6

[a] data from 6 days of cultivation were used for calculation.

Aeration with 10% (v/v) CO_2 also caused a downward shift of pH of about 1.5-2 in the culture medium. As CO_2 dissolves in water; algae cells can use it for the photosynthesis process to promote all types of growth. Utilization of a higher concentration of carbon dioxide results in reducing the pH since the residual CO_2 that is not used for the formation of biomass will be converted to carbonic acid [264].

5.3.2. Effect of different nitrogen sources on growth

A wide variety of nitrogen sources such as nitrate, nitrite, ammonia, urea and yeast extract, can be used for growing microalgae [265]. In this experiment, KNO_3, $NaNO_3$, NH_4NO_3 and yeast extract were used to investigate the effect of nitrogen source on microalgae growth. As shown in Fig. 5-4, the initial growth of *Chlorococcum* sp. in BG-11 medium supplemented with yeast extract at an early lag phase seems similar to other nitrogen sources. On the other hand, yeast extract was the best nitrogen source. The three tested inorganic nitrogen sources were similar in terms of microalgae growth. After ten days of cultivation, the maximum biomass obtained were approximately 0.16 g L^{-1} (KNO_3), 0.19 g L^{-1} ($NaNO_3$), 0.18 g L^{-1} (NH_4NO_3) and 0.45 g L^{-1} (yeast extract).

The final biomass content was similar among the three tested inorganic nitrogen sources, but the highest biomass was obtained from the culture with $NaNO_3$. For further experiments, sodium nitrate was selected as a nitrogen source because it is a nitrogen source in the BG-11 medium; besides, it is less costly than yeast extract.

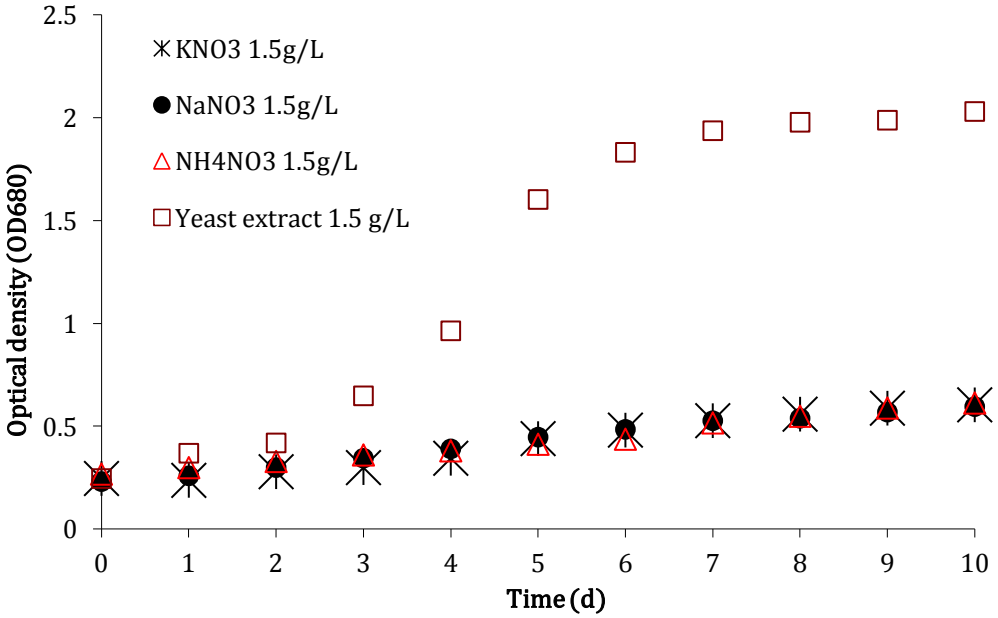


Fig. 5-4. Time course of cell growth of *Chlorococcum* sp. in BG-11 medium with different nitrogen sources.

5.3.3. Effect of yeast extract concentration on growth, biomass and lipid content

The BG-11 medium containing 0.1, 1.5, 3, and 6 g L^{-1} of yeast extract were used to investigate the effect of yeast extract in *Chlorococcum* sp. The time course profiles of cell growth obtained with different yeast extract concentrations are shown in Fig. 5-5. As shown in Fig. 5-5, the growth of *Chlorococcum* sp. increased significantly with increasing yeast extract concentration. The best growth in yeast extract media was between 0 and 7 days of cultivation, after which cells began to die.

Table 5-3 shows the biomass concentration in dry weight and lipid content (% of dry weight) obtained at different yeast extract concentrations. It is clear that the lipid content decreased when the concentration of yeast extract increased. Results showed that the optimal yeast extract concentration for lipid accumulation was 1.5 g L^{-1}. Hence, yeast extract was not selected for further cultivation because it is too costly for microalgae cultivation. The lipid productivities were 6, 12, 10 and 5 mg $L^{-1}d^{-1}$ at 0.1, 1.5,3 and 6 g L^{-1} of yeast extract concentration, respectively.

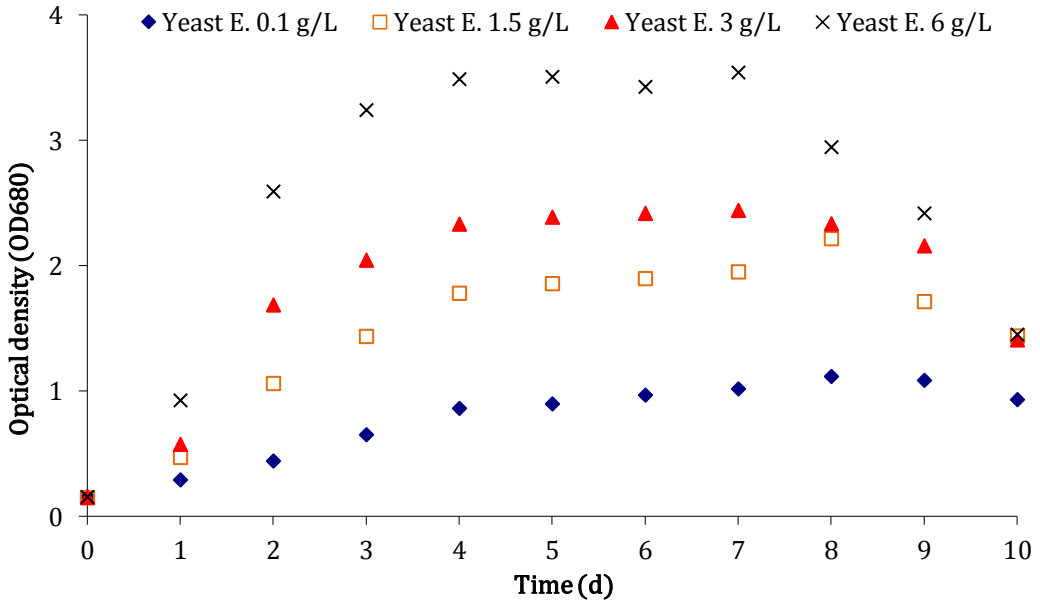


Fig. 5-5. Growth of *Chlorococcum* sp. in the BG-11 medium with different yeast extract concentrations.

Table 5-3. Biomass and total lipid content of *Chlorococcum* sp. in the BG-11 medium with different yeast extract concentrations.

BG-11 medium (Yeast extract g L^{-1})	Biomass (g L^{-1})	Total lipid (% of dry weight)
0.1	0.26 ± 0.01	23.9 ± 1.0
1.5	0.45 ± 0.02	26.1 ± 0.5
3.0	0.44 ± 0.01	21.8 ± 0.8
6.0	0.45 ± 0.01	12.2 ± 0.4

5.3.4. Effect of nitrate concentration

The BG-11 medium containing nitrate from 0.2 to 35.3 mM was used to investigate the effect of nitrogen concentration on biomass growth and lipid content. As presented in Fig. 5-6, the biomass achieved 0.26, 0.51, 0.48, 0.63 and 0.47 g L^{-1}, when using 0.2, 1.2, 8.8, 17.7 and 35.3 mM of nitrate, respectively. Under these conditions, the lipid content decreased significantly from 43, 32, 18 and 13% to 9% of dry weight. The lipid mass was 110, 170, 90, 80 and 40 mg L^{-1}, respectively. In the following experiments, a concentration of 1.2 mM nitrate was selected as the optimum initial nitrate concentration because the highest lipid mass (170 mg L^{-1}) was achieved. A decrease in nitrate concentration led to a decrease in the biomass concentration; in

contrast, a decrease in nitrate concentration led to an increase in lipid content [150]. A number of researchers reported that nitrogen concentration is a major factor affecting lipid accumulation in algae [110, 150, 151, 155, 176].

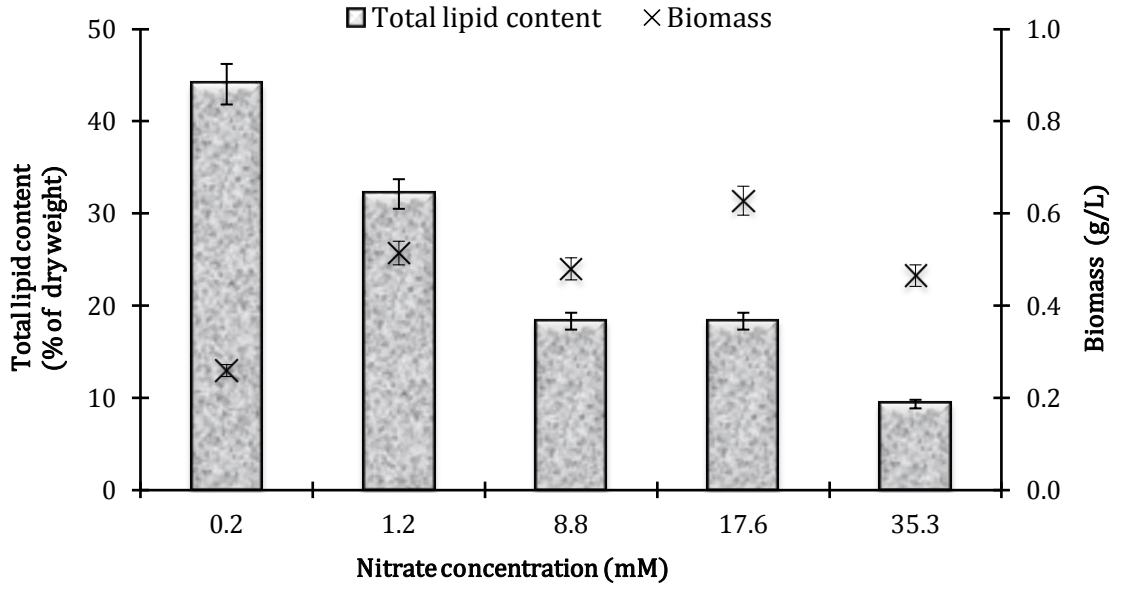


Fig. 5-6. Total lipid content and biomass concentration of *Chlorococcum* sp. at different levels of nitrate.

The amount of nitrate in the medium affected the growth of cells and biosynthesis of lipids. Increasing total lipid content was in accordance with decreasing nitrate concentration. These results are in agreement with other studies, which reported that lipid content increased under nitrogen starvation. Microalgae species such as *Chlorella* sp. [150], *Neochloris oleoabundans* [151], *Nannochloropsis oculata* [154], *Nannochloropsis* sp. [176] increased lipid content between 1.5 to 5 times under nitrogen starvation. However, microalgae species with a high lipid content commonly grow slowly. The increased lipid content of the algae does not induce an increase in overall productivity of lipids because higher lipid content is often found where there is low biomass productivity [110].

5.3.5. Acetate and glucose as carbon source on growth and lipid content

The modified BG-11 medium supplemented with acetate and glucose was used to investigate the effect of carbon source on lipid content in cells of *Chlorococcum* sp. The time course profiles of cell growth obtained are shown in Fig. 5-7. Growth of *Chlorococcum* sp. improved significantly when the medium contained acetate or glucose.

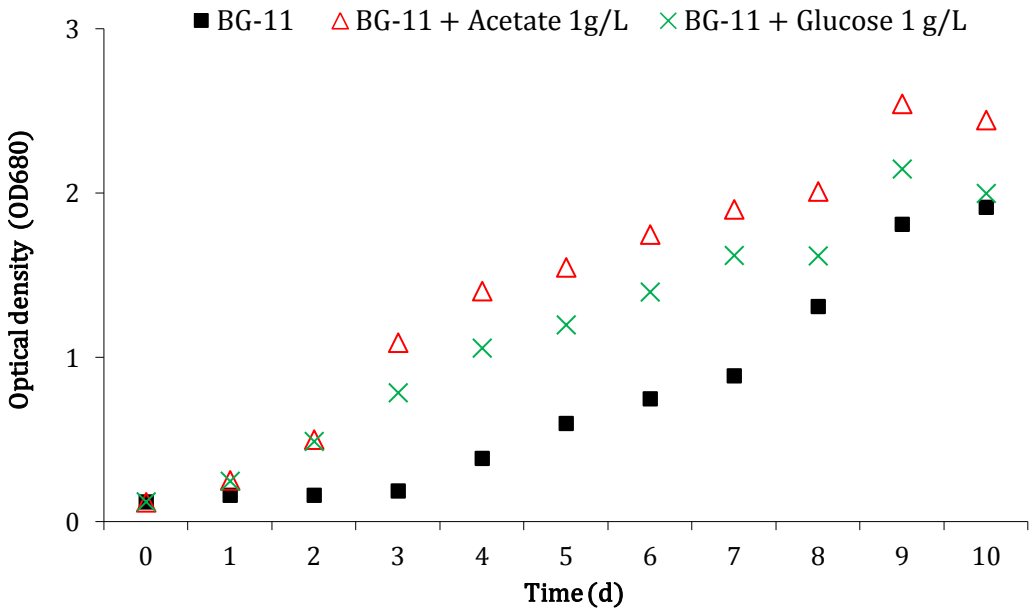


Fig. 5-7. Time course of cell growth of *Chlorococcum* sp. in the modified BG-11 medium with acetate and glucose as carbon source.

Table 5-4 shows the biomass concentration and lipid content in the modified BG-11 medium. The lipid content increased when the microalgae cells were grown under mixotrophic conditions. The results show that acetate and glucose in the medium were able to enhance the lipid concentration in the microalgae by about 16.1% and 7.7%. It was obvious that cultivation with acetate and glucose (mixotrophic conditions) has proven to be a good strategy for obtaining more biomass and a high lipid content in *Chlorococcum* sp. However, acetate was a better candidate than glucose as an organic carbon source to enhance lipid content. Acetate and glucose are known as precursors for lipid synthesis (fatty acid). Acetate is taken up by microorganisms and converted to Acetyl *CoA* in the cytoplasm. Afterwards, the acetyl *CoA* is used for fatty acid synthesis [164]. Glucose is also the major source of acetyl CoA for fatty acid synthesis. Glucose is degraded to pyruvate by aerobic glycolysis in the cytoplasm. Then, pyruvate is transported into the mitochondria forming acetyl CoA, which is used for fatty acid synthesis [266]. A number of microalgae species are able to grow under mixotrophic [173, 178] or heterotrophic conditions [107, 256]. The presence of an organic carbon source in the medium could increase the biosynthesis of lipid in algae cells [107, 173, 178, 256]. Acetate, glucose, glycerol, and sucrose are used the most frequently as a source of carbon. Organic carbons are able to trigger a lipid accumulation in algae cells, whereby the carbon source cannot be used for the protein synthesis, but can be used for lipid synthesis [173].

Table 5-4. Biomass and total lipid content of *Chlorococcum* sp. in the modified BG-11 medium supplemented with acetate and glucose as carbon source.

Medium	Biomass (g L^{-1})	Total lipid (% of dry weight)
BG-11	0.58 ± 0.05	29.3 ± 2
BG-11 (Acetate 1 g L^{-1})	0.84 ± 0.01	45.4 ± 2
BG-11 (Glucose 1 g L^{-1})	0.77 ± 0.03	37.0 ± 1

The lipid accumulation of cells in the different media was assessed with Nile red dye using a fluorescence microscope; the lipid droplets displayed a yellow color (Fig. 5-8).

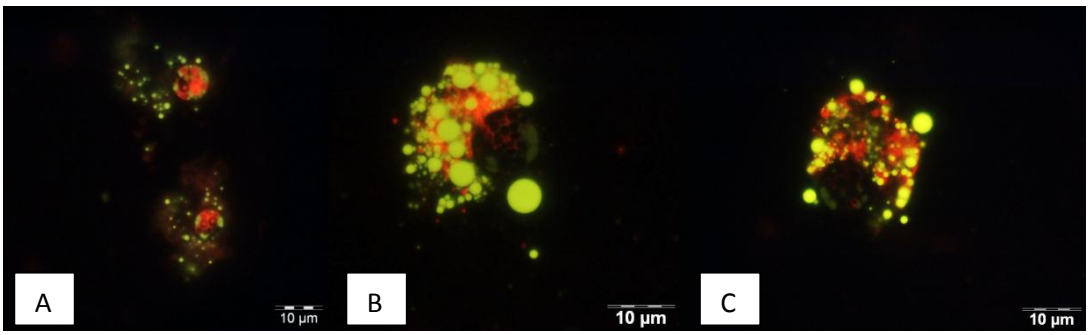


Fig. 5-8. *Chlorococcum* sp. cells under a fluorescence microscope in (A) the BG-11 medium, (B) modified BG-11 medium with acetate and (C) modified BG-11 medium with glucose.

5.3.6. Effect of acetate concentration and inoculum concentration

Concentrations from 0 to 280 mM acetate were used to investigate the effect of acetate as a carbon source on biomass and lipid production. Cells sizes were 5.45–19.08 µm in a BG-11 medium supplemented with acetate as a carbon source, and the cell sizes were 4.65–8.58 µm in BG-11 medium without carbon source. The final pH ranged from 7.8 to 8.0 under photoautotrophic conditions (in the BG-11 medium without carbon source). Biomass concentration and total lipid contents were measured after 10 days of incubation. The pH value increased during growth when the acetate was used as carbon source. The final pH value ranged from 9.0 to 10.0 at different levels of acetate.

The flask culture image in Fig. 5-9 shows that the cells of *Chlorococcum* sp. under photoautotrophic conditions were still green and did not cause the loss of the green

color, namely chlorophyll. This was obviously different to mixotrophic cultures where the cultivation broth will turn completely yellow due to the loss of chlorophyll.

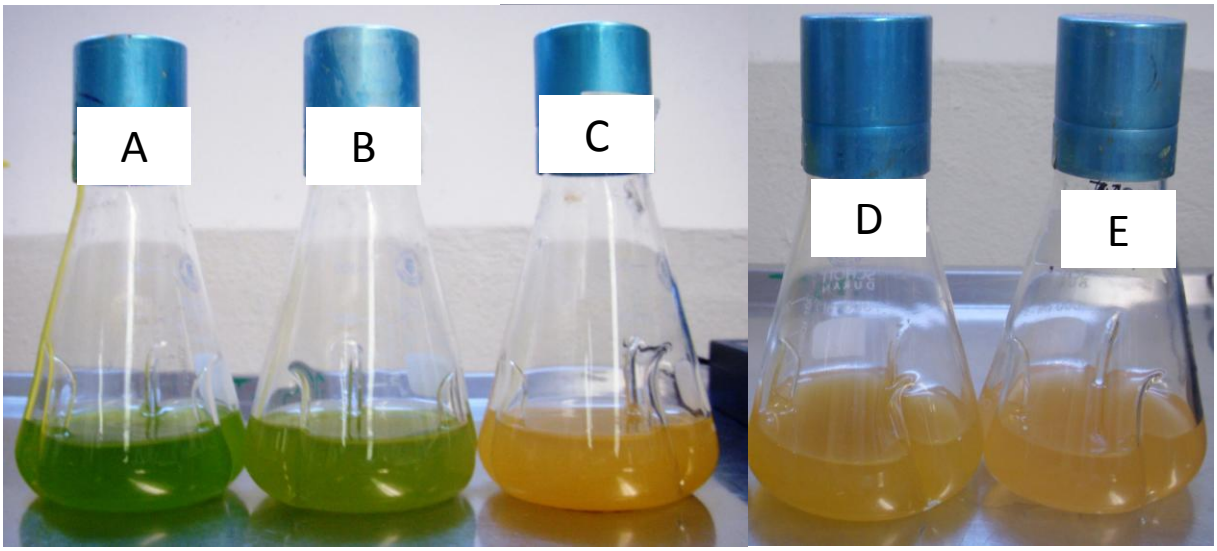


Fig. 5-9. Photoautotrophic and mixotrophic cultures of *Chlorococcum* sp. (A: photoautotrophic; B: acetate 7 mM; C: acetate 70 mM; D: acetate 140 mM and E: acetate 280 mM).

Table 5-5 shows the different concentrations of acetate and the resulting total lipid content and lipid productivity at different concentrations of inoculum. Two concentrations (10^6 cells/ml and 10^7 cells/ml) were used. This experiment was carried out under photoautotrophic and mixotrophic conditions. In different levels of acetate with an inoculum concentration of 10^6 cells/ml, the maximum biomass concentration (0.87 g L^{-1}), total lipid content (47.2% of dry weight) and lipid productivity (41.1 mg L^{-1} d^{-1}) were attained at 70 mM acetate. When the inoculum concentration in the medium was increased 10-fold, a higher lipid content could be achieved. Under photoautotrophic conditions, lipid productivity increased 5-fold (from 10 to 50 mg $L^{-1}d^{-1}$) when the inoculums concentration was 10-fold. Under mixotrophic conditions, the increase in concentration of inoculums was also accompanied by an increase of total lipid content at an acetate concentration of 70 mM; total biomass achieved was 1.75 g L^{-1}; total lipid content increased 1.4-fold (from 47.2% to 51.9% of dry weight; and the lipid productivity increased 2.2-fold (from 41.1 to 90.9 mg $L^{-1}d^{-1}$). Table 5-5 describes how relatively high acetate concentrations reduced the lipid productivity. These results indicate that *Chlorococcum* sp. was sensitive to higher concentrations of acetate. The results indicate that the concentration of acetate and inoculum affected the total lipid

content and lipid productivity, thus an inoculum concentration of 10^7 cells ml^{-1} and a concentration of acetate 70 mM were used for further experiments. Heredia-Arroyo et *al*. [267] and Abreu et *al*. [258] reported that *Chlorella vulgaris* could grow in photoautotrophic, mixotrophic and heterotrophic conditions; and the mixotrophic cultivation especially could produce more cell biomass than the photoautotrophic or heterotrophic cultures individually or combined. Li et *al*. [255] also observed that cultivation under mixotrophic conditions for *Chlorella minutissima* UTEX 2341 enhanced biomass and lipid productivity. Results show that cultivation under mixotrophic conditions contributed to a higher lipid production than under photoautotrophic conditions. These results are in agreement with previous studies [178, 255, 258, 267].

Table 5-5. Total lipid content and lipid productivity of *Chlorococcum* sp. at different concentrations of acetate and at different concentrations of inoculum.

Acetate concentration (mM)	A^a	B^a	A^b	B^b
0	26.2 ± 3.1	10.0 ± 1	37.7 ± 1.7	50.2 ± 2
7	33.2 ± 2.9	15.9 ± 2	37.2 ± 1.3	44.2 ± 2
70	47.2 ± 0.9	41.1 ± 2	51.9 ± 2.1	90.9 ± 4
140	41.3 ± 1.1	29.7 ± 1	44.1 ± 2.0	68.2 ± 3
280	33.7 ± 1.6	15.2 ± 1	35.6 ± 1.8	37.2 ± 2

A: Total lipid content (% of dry weight), B: Lipid productivity (mg $L^{-1}d^{-1}$)
[a] Inoculum concentration of 10^6 cell/ml, [b] Inoculum concentration of 10^7 cell/ml

5.3.7. Effect of photoperiod on biomass, total lipid and fatty acid content

The acetate level at 70 mM and nitrate at 1.2 mM were used to investigate the biomass concentration and lipid content of *Chlorococcum* sp. on a 2-L scale with a photoperiod of LD 12:12 h and LD 24:0 h. Fig. 5-10 shows profiles of biomass, total lipid content and substrate consumption during the cultivation of *Chlorococcum* sp. under photoautotrophic and mixotrophic conditions. Under photoautotrophic conditions the maximum biomass content (1.18 g L^{-1}) was obtained when the culture was at LD 24:0. Total lipid content was not significantly different from cultivation at LD 24:0 h and LD 12:12 h and total lipid was about 43.2% and 44.3% of dry weight at the end of cultivation, respectively. Under mixotrophic conditions the maximum biomass content

(1.52 g L^{-1}) was obtained when the culture was exposed to 12 h light and 12 h dark over 9 days of cultivation. The maximum total lipid content was equal at LD 24:0 h and LD 12:12 h and the total lipid content was about 56.2% of dry weight under both conditions over 9 days of cultivation. Whereas the maximum biomass (1.45 g L^{-1}) was slightly lower at LD 24:0 h than LD 12:12 h. No significant effect of photoperiod was observed on biomass concentration and total lipid content under photoautotrophic conditions. In contrast, a photoperiod has a significant effect on biomass and total lipid content in *Nannochloropsis* sp. [187] and in *Chlorella vulgaris* [268]. The total lipid content for a given species depends on the growth phase. The lowest yield occurs in the logarithmic phase, with an increase in the late logarithmic phase, before stabilization or an increase in the stationary phase [269]. The pH values in 4-day cultivations were 9.4 and 8.9 at LD 12:12 h and LD 24:0 h, respectively. The pH values under photoautotrophic conditions were relatively equal (pH 7.8 – 8.0) with the initial pH (adjusted at pH 8.0 before autoclaving). Acetate uptake was 46.0% at LD 12:12 h and 63.3% at LD 24:0 h. The beginning nitrate starvation was observed on day 7 of cultivation and day 4 of cultivation for phosphate starvation (Fig. 5-10). Nitrate and phosphate starvation triggered total lipid accumulation.

Table 5-6 shows the summary of fatty acids composition, biomass, total lipid and total fatty acid content of *Chlorococcum* sp. after 14 days of incubation at LD 12:12 h and LD 24:0 h. The fatty acid methyl ester composition was analyzed under conditions of nitrate and phosphate starvation after 14 days of cultivation. Total fatty acid content was achieved around 21-26 % of the dry weight to represent 43-56% of total lipid in biomass. The major components of fatty acids in *Chlorococcum* sp. were C16:0, C18:0, C18:1, C18:2 and C18:3, with a minor fraction of C16:1. In this study, the fatty acid composition showed no significant differences at different photoperiods under photoautotrophic and mixotrophic conditions. This result was different from Khoeyi et *al*. [268] who found that photoperiods have a significant effect on algae lipid composition in *Chlorella vulgaris*.

Table 5-6. Summary of fatty acid composition and total fatty acid content of *Chlorococcum* sp. after 14 days of incubation with a photoperiod of LD 12:12 h and LD 24:0 h.

Fatty acid	Fatty acid composition (%)			
	A [a]	B [a]	C[b]	D [b]
C16:0	32.2 ± 0.0	30 ± 0.1	31.0 ± 0.2	32.2 ± 0.6
C16:1	2.2 ± 0.0	2 ± 0.0	2.1 ± 0.1	2.2 ± 0.1
C18:0	5.4 ± 0.0	6.2 ± 0.2	5.2 ± 0.1	5.6 ± 0.1
C18:1	34.2 ± 0.0	32.5 ± 0.4	38.3 ± 0.3	37.1 ± 1.7
C18:2	11.6 ± 0.0	13.8 ± 0.0	10.3 ± 0.0	10.3 ± 0.1
C18:3	8.3 ± 0.0	8..6 ± 0.0	7.0 ± 0.0	7.2 ± 0.1
Unknown	5.9 ± 0.0	6.7 ± 0.1	6.2 ± 0.0	5.5 ± 0.1
ΣSaturated (Sat)	36.4	36.2	36.2	37.8
ΣUnsaturated (Un)	63.6	57.1	63.8	56.7
ΣUn/ ΣSat	1.7	1.6	1.8	1.5
Fatty acid content [c]	23.2 ± 0.7	21.0 ± 1.5	28.1 ± 1.3	25.3 ± 2.2

[a] A and B, cultivations under photoautotrophic conditions with a photoperiod of LD 12:12 h and LD 24:0 h.
[b] C and D, cultivations under mixotrophic conditions with a photoperiod of LD 12:12 h and LD 24:0 h.
[c] Fatty acid content in % of dry weight

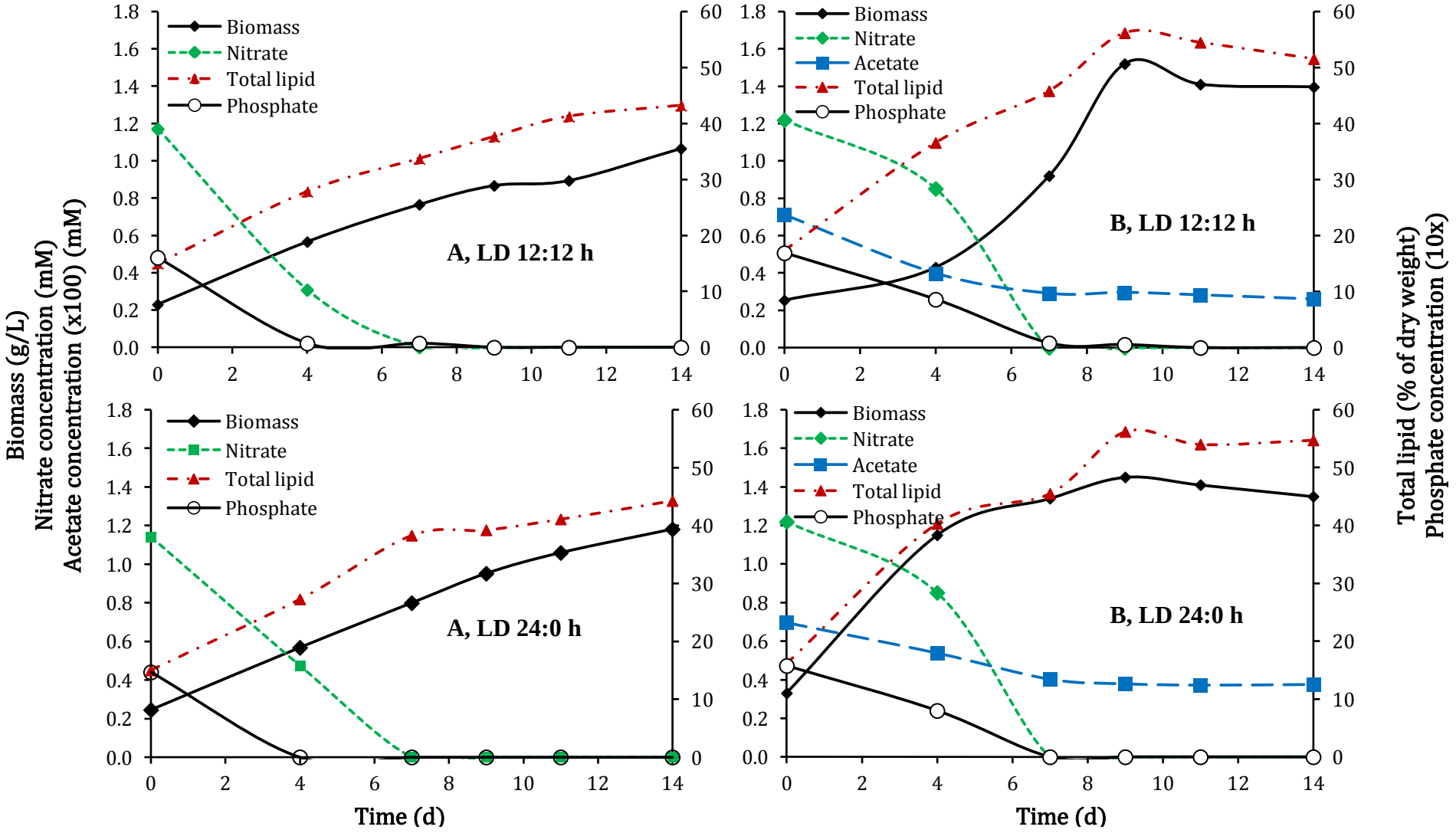


Fig. 5-10. Profiles of biomass and total lipid content of *Chlorococcum* sp. (A) under photoautotrophic conditions and (B) under mixotrophic conditions during progressive nitrate and phosphate starvation with a photoperiod of LD 12:12 h and LD 24:0 h.

5.3. 8. Effect of nitrate starvation on fatty acid content

To observe the effect of nitrate starvation (nitrate deficiency) on fatty acid content, photoautotrophic and mixotrophic conditions with a photoperiod LD 12:12 h were used. Fig. 5-11 shows the influences of nitrate starvation on fatty acid content and profile of *Chlorococcum* sp. under different conditions. As observed, fatty acid content increased after nitrate starvation for both conditions. Fatty acid content increased 2.9-fold under photoautotrophic and 4.6-fold under mixotrophic conditions after 9 days of cultivation, where the increase (in % of dry weight) was due to the increase of all fatty acids. Under photoautotrophic conditions an increase (% total of fatty acids) was: C16:0 from 1.7% to 7.5%, C16:1 from 0.3% to 0.5%, C18:0 from 0.4% to 1.3%, C18:1 from 1% to 8%, C18:2 from 1.3% to 2.7%, C18:3 from 0.7% to 1.9%, and unknown fatty acids from 0.6% to 1.4%. Whereas an increase was C16:0 from 1.8% to 8.7%, C16:1 from 0.2% to 0.6%, C18:0 from 0.2% to 1.5%, C18:1 from 1.5% to 10.8%, C18:2 from 1.5% to 2.9%, C18:3 from 0.8% to 2% and unknown fatty acids from 0.6% to 1.7% under mixotrophic conditions.

Under photoautotrophic conditions nitrate starvation increased fatty acid content (% total of fatty acids): C16:0 from 28.7% to 32.2%, C18:1 from 17.4 to 34.2% and decreased C16:1 from 4.1% to 2.2%, C18:0 from 6.8% to 5.4%, C18:2 from 21.1% to11.6%, C18:3 from 11.8% to 8.3% and unknown fatty acids from 10.15 to 5.9%. In the nitrogen starvation phase, the fatty acid composition under mixotrophic conditions showed a similar trend as cultivation under photoautotrophic conditions. Nitrate starvation increased fatty acid C16:0 from 28.7% to 31.2%, C18:1 from 17.4 to 38.2% and decreased C16:1 from 4.1% to 2.1%, C18:0 from 6.8% to 5.3%, C18:2 from 21.1% to10.2%, C18:3 from 11.8% to 6.7% and unknown fatty acids from 10.1% to 6.2%. Afterwards, oleic acid (C18:1) and palmitic acid (C16:0) content increased with increasing culture time and became the predominant fatty acids at the end of cultivation. An increased accumulation of C18:1 under nitrogen starvation was reported in *Nannochloropsis* sp. [176], *Chlorella minutissima* UTEX 2341 [169], *Nannochloropsis oculata* [154]. Otherwise, the percentage of palmitoleic acid (C16:1), linoleic acid (C18:2), linolenic acid (C18:3) and unknown fatty acids decreased with culture time. Nitrate starvation has a tendency to accumulate C16:0 and C18:1, high oleic acid (C18:1) was followed by a low content of C18:2 and C18:3 [169, 176]. During logarithmic growth, most lipids are glycerol-based polar membrane lipids which function to

maintain cells structures [164]. Unlike the glycerolipids, triacylglycerols (TAG) are neutral lipids used for storage without structural function. TAG production is enhanced under photo-oxidative stress or nutrient starvation [164]. Hu et *al.*, 2008 [164] reported that the increase in the relative proportions of both saturated and monounsaturated fatty acids (C16:0 and C18:1) and the decrease in the proportion of polyunsaturated fatty acids in the total lipid content are also associated with growth-phase transition from the logarithmic to the stationary phase.

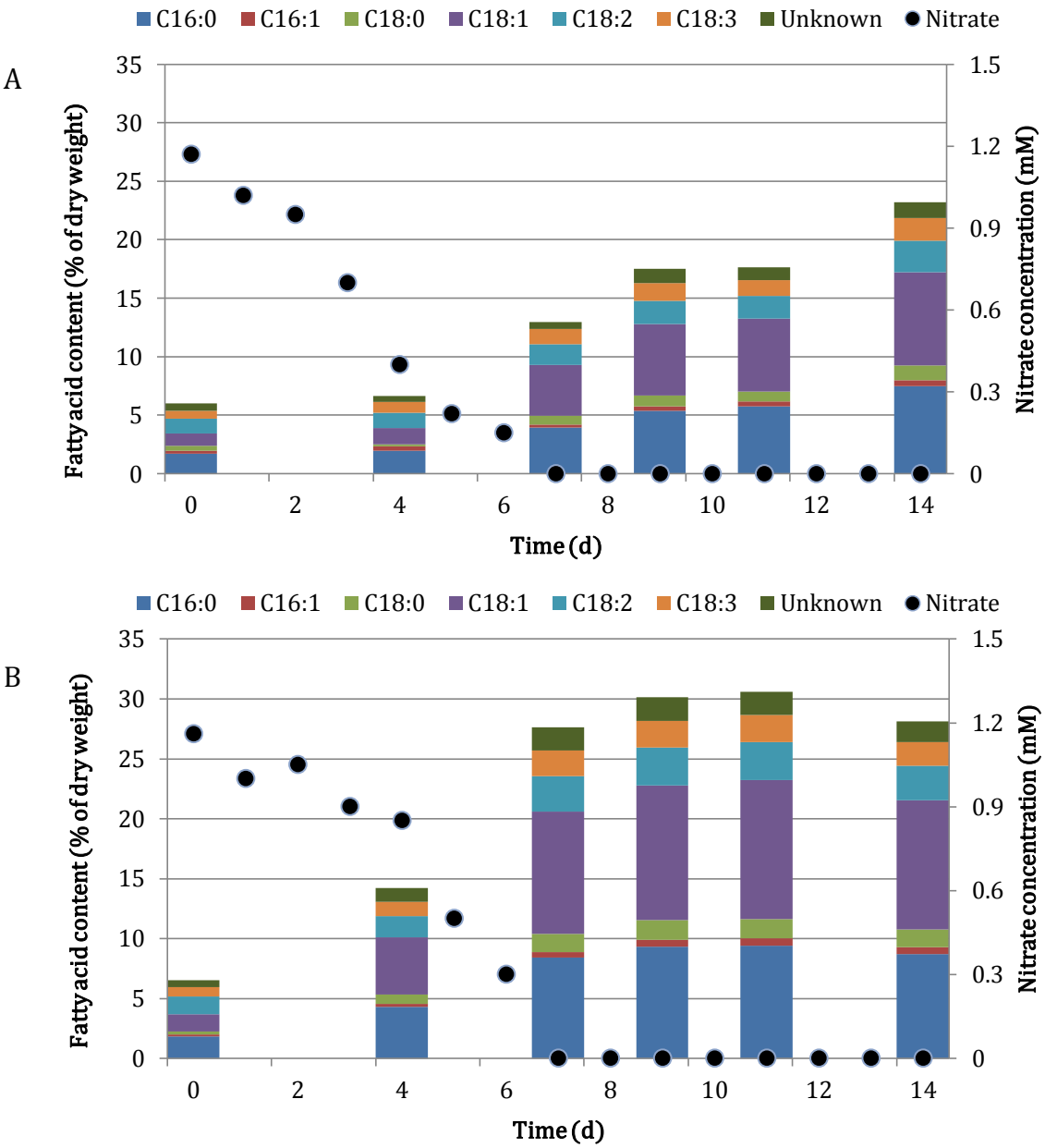


Fig. 5-11. Influences of nitrate starvation on fatty acid content and profile of *Chlorococcum* sp. (A) under photoautotrophic conditions and (B) under mixotrophic conditions with a photoperiod of LD 12:12 h.

5.3.9. Effect of different types of salt on biomass, lipid and fatty acid content

The effect of different types of salt on biomass, lipid and fatty acid production was investigated. Sodium chloride and KCl were used for cultivation of *Chlorococcum* sp. As shown in Fig. 5-12 the highest biomass content (0.78 g L^{-1}) was reached in the medium without additional salt supplement. The biomass contents were slightly lower at the end of cultivation under modified BG-11 medium with NaCl and KCl, 0.76 g L^{-1} and 0.76 g L^{-1}, respectively. Table 5-7 shows that the type of salt did not affect the biomass productivity in *Chlorococcum* sp. The acetate concentrations at the end of cultivation were similar to those at the beginning.

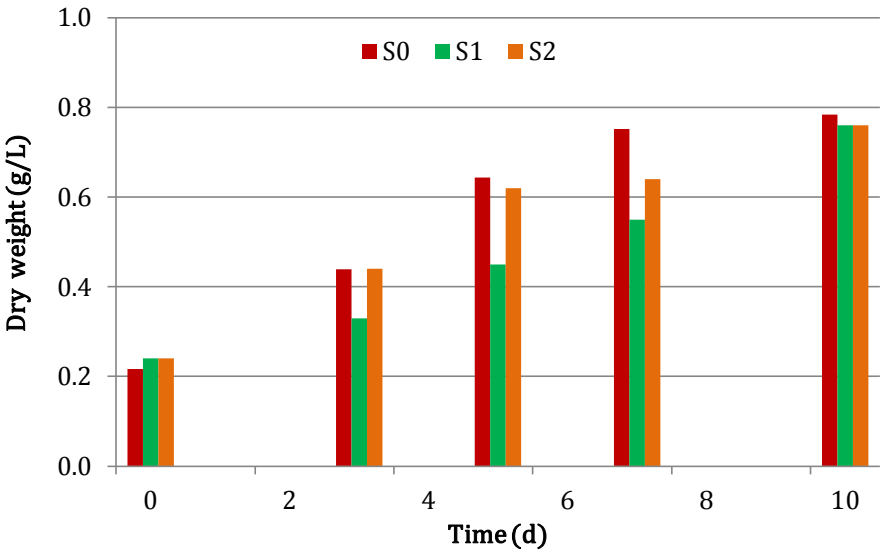


Fig.5-12. Biomass content of *Chlorococcum* sp. in different media. S0, the modified BG-11 medium; S1, the modified BG-11 medium supplemented with NaCl 1% (w/v); S2, the modified BG-11 medium supplemented with KCl 1% (w/v).

The lipid content in the NaCl medium was slightly lower than the lipid content in the KCl medium. However, the highest lipid productivity was reached in the modified BG-11 medium without additional salt. The highest fatty acid production was reached when algae cells were grown in the modified BG-11 medium supplemented with KCl. Results show that the presence of KCl was more beneficial to accumulating fatty acids (Table 5-7). The relative contents of lipid composition were in the same range in NaCl medium and KCl medium. Oleic acid (C18:1) was slightly higher in the medium supplemented with KCl. The percentage of C18:3 was highest than in NaCl medium. The effect of potassium chloride on biomass growth, lipid content and fatty acid composition

in microalgae cells is yet unreported. A number of papers reported the effect of salinity (NaCl) on the biochemical composition of microalgae species [182, 183, 270].

Table 5-7. Summary of fatty acid composition, total lipid content, lipid productivity and biomass productivity of *Chlorococcum* sp. with different salts after 10 days of cultivation. S0, BG-11 medium; S1, BG-11 medium containing NaCl 1% (w/v); S2, BG-11 medium containing KCl 1% (w/v).

	S0	**S1**	**S2**
Fatty acid composition:			
C16:0	26.2 ± 1	23.9 ± 0.2	24.5 ± 1
C16:1	2.3 ± 0.1	1.9 ± 0	2.1 ± 0.1
C18:0	5.6 ± 0.2	4.7 ± 0	5.8 ± 0
C18:1	34.2 ± 2	30.4 ± 1	35.2 ± 1
C18:2	8.5 ± 0.2	8.9 ± 0	9.3 ± 0
C18:3	13.9 ± 0.3	15.2 ± 0	11.5 ± 0.2
Unknown fatty acids	9.3 ± 0.1	14.9 ± 0.2	11.5 ± 0
Total lipid content			
(% of dry weight)	24.7 ± 0	22.2 ± 0.7	24.7 ± 0.5
Lipid productivity (mg $L^{-1}d^{-1}$)	19.4 ± 1	11.6 ± 0.1	12.8 ± 0.2
Biomass productivity (mg $L^{-1}d^{-1}$)	67.5 ± 5	52.0 ± 3	52.0 ± 6
Fatty acid content (% of dry weight)	16.9 ± 1	15.6 ± 0.5	23.9 ± 1

5.3.10. Effect of CO_2 aeration on biomass, lipid productivity and fatty acid content

Chlorococcum sp. can be grown in CO_2-enriched air and the effect of CO_2 aeration on biomass, lipid productivity and fatty acid content was investigated in the modified BG-11 medium under photoautotrophic conditions (Table 5-8). The maximum total lipid content under cultivation in CO_2-enriched air (6% v/v) medium was 30% of dry weight. Culture aerated by CO_2-enriched air resulted in a productivity increase of 1.5-fold in biomass and 1.7-fold in lipid productivity when compared to culture with air aeration. Aeration with CO_2 caused a downward shift of pH of about 0.95-1.13 in the culture medium. An increasing concentration of carbon dioxide enhanced growth and the total lipid content. These results are in agreement with the data reported by Widjaja et *al.* [177]. Fatty acid content under CO_2 aeration was slightly higher than culture under air

aeration. The relative content of lipid composition was fundamentally the same for both air aeration and CO_2 aeration. The percentage of C16:0 under CO_2 aeration was slightly higher than under air aeration. Little information is available on the effect of carbon dioxide on the fatty acid composition. However, Tsuzuki et *al*. [271] reported that the degree of unsaturated fatty acids is higher in *Chlorella vulgaris* cells grown with air than in the cells grown with air enriched with 2% (v/v) CO_2, and that the change in the ratio of linoleic acid (C18:2) to α-linolenic acid (C18:3) is particularly significant.

Table 5-8. Summary of fatty acid composition, total lipid content, lipid productivity and biomass productivity of *Chlorococcum* sp. under aerated with air and CO_2-enriched air (6% v/v).

	Aerated with air	CO_2-enriched air
Fatty acid composition:		
C16:0	26 ± 1	31 ± 2
C16:1	2 ± 0.1	2 ± 0
C18:0	6 ± 0	4 ± 0.2
C18:1	34 ± 1	31 ± 1
C18:2	9 ± 0.2	10 ± 0
C18:3	14 ± 1	13 ± 1
Unknown fatty acids	9 ± 0.1	10 ± 0
Total lipid content (% of dry weight)	23 ± 0	30 ± 0.7
Lipid productivity (mg $L^{-1}d^{-1}$)	22 ± 1	37 ± 1
Biomass productivity (mg $L^{-1}d^{-1}$)	81 ± 4	123 ± 2
Fatty acid content (% of dry weight)	17 ± 1	23 ± 0.3

5.3.11. Effect of light intensity on biomass, lipid productivity and fatty acid content

The effect of light intensity on biomass and fatty acid content is shown in Fig. 5-13. Light intensities of 100, 250 and 500 μmol photons $m^{-2}s^{-1}$ were used. Fig. 5-13 shows that photo-inhibition on biomass growth was observed in different media at the end of cultivation. *Chlorococcum* sp. produced the maximum biomass and maximum total fatty acid under photoautotrophic conditions at low light intensity. No significant pH shift was found under photoautotrophic conditions. Otherwise, when acetate was used as a carbon source, an upward shift of pH by about 1.5-2 units in the culture medium was

observed. Under low light intensity acetate uptake was between 72-76 %, while under medium and high light intensity cells had similar acetate utilization. However, the cell uptake of acetate was very low.

Chlorococcum sp. under mixotrophic conditions showed the same trend as cultivation under photoautotrophic conditions (M1); cells also produced the highest amount of biomass and fatty acid under low light intensity. The fatty acid content under mixotrophic conditions (M2 and M3) at different light intensities showed no significant differences in the amount of fatty acids (% of dry weight) at the end of cultivation. However, an inhibition of fatty acid production occurred under high light intensity and photoautotrophic conditions (M1). Cultivation under low light and in the modified BG-11 medium supplemented with acetate led to higher biomass and lipid productivity at the end of cultivation (Table 5-9). Based on the results, cultivation of *Chlorococcum* sp. under high light intensity resulted in lower biomass and lipid productivity in all media than under low light irradiance. The presence of salt in the medium also reduced biomass and lipid productivity. At high light intensity, the photosynthetic capacity was reduced, which was indicated by reducing biomass production.

Fatty acid composition changed at different light intensities under photoautotrophic conditions; the percentage of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) decreased with increasing light intensity, whereas polyunsaturated fatty acids (PUFA) increased with increasing light intensity. On the other hand, the results for fatty acid composition under mixotrophic conditions showed that an increase in light intensity was related to reduced MUFA and increased SFA and PUFA. Light intensity influenced biomass productivity and fatty acid composition under both conditions (photoautotrophic and mixotrophic). These results are in agreement with those reported by Khoeyi et *al*. [268] showing that the light intensity causes significant differences in biomass and fatty acid composition.

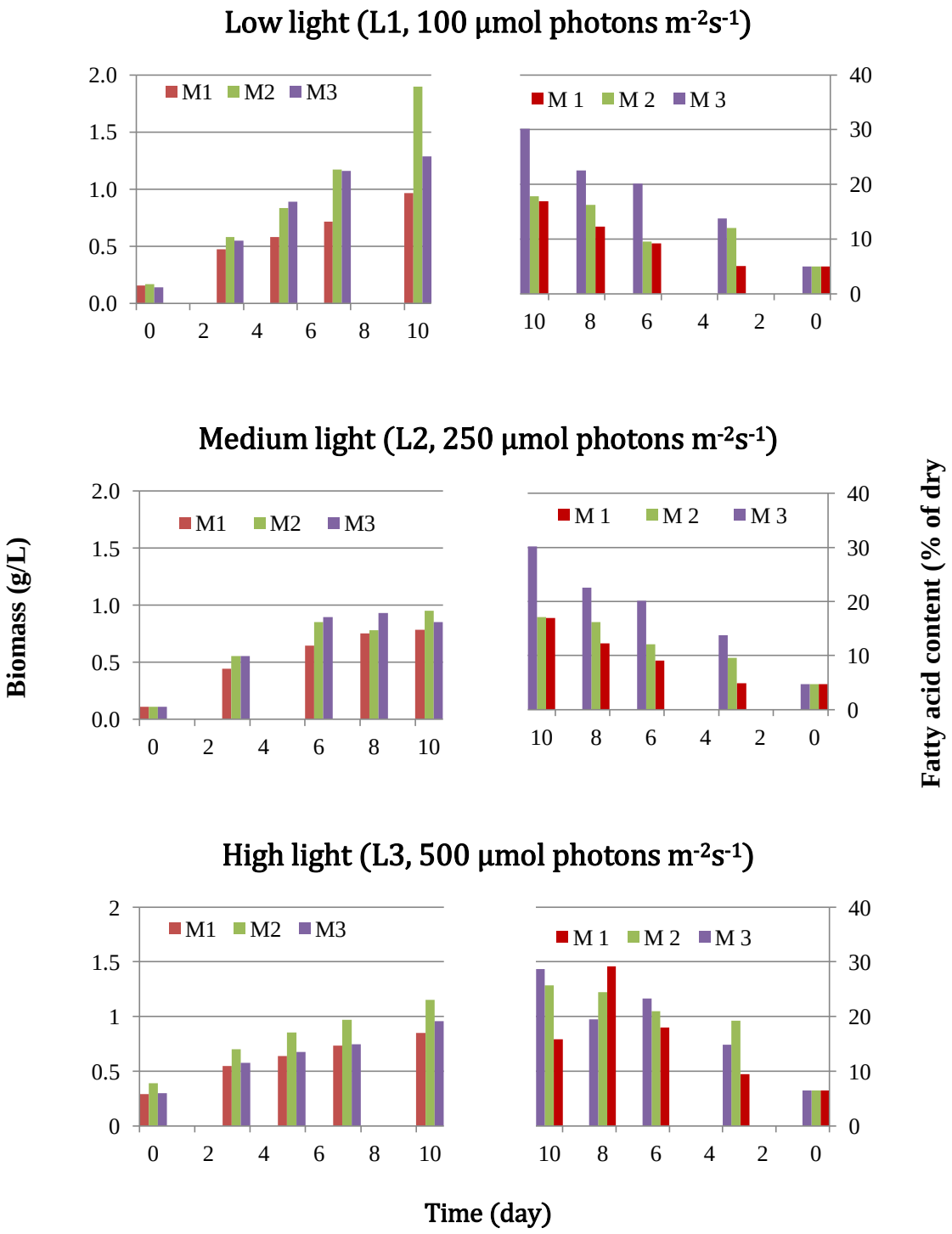


Fig. 5-13. Biomass and fatty acid content of *Chlorococcum* sp. in different media under different light intensities. L1, 100 µmol photons $m^{-2}s^{-1}$; B, L2, 250 µmol photons $m^{-2}s^{-1}$; C, L3 500 µmol photons $m^{-2}s^{-1}$; M1, modified BG-11 medium; M2, modified BG-11 medium supplemented with acetate 70 mM; M3, modified BG-11 medium supplemented with acetate 70 mM and NaCl 1% (w/v).

Table 5-9. Summary of fatty acid composition and total lipid of *Chlorococcum* sp. with three levels of light intensities.M1, modified BG-11 medium; M2, modified BG-11 medium supplemented with acetate 70 mM; M3, modified BG-11 medium supplemented with acetate 70 mM and NaCl 1% (w/v).

	L1, 100 µmol photons $m^{-2}s^{-1}$			L2, 250 µmol photons $m^{-2}s^{-1}$			L3, 500 µmol photons $m^{-2}s^{-1}$		
	M1	M2	M3	M1	M2	M3	M1	M2	M3
Fatty acid composition:									
C16:0	27.2	27.1	26.4	26.2	27.5	24.9	28.9	30.0	27.9
C16:1	1.9	3.3	2.8	2.3	3.0	2.8	1.8	2.7	2.9
C18:0	6.2	6.1	6.6	5.6	5.5	6.5	3.4	5.3	4.8
C18:1	32.0	39.0	39.7	34.2	37.3	38.9	22.6	33.6	36.4
C18:2	8.7	7.6	8.4	8.5	7.7	8.1	9.3	9.1	9.6
C18:3	13.9	9.1	8.5	13.9	10.6	8.7	18.4	10.1	10.2
Unknown fatty acids	10.2	7.8	7.7	9.3	8.4	10.1	15.5	9.2	8.1
Saturated fatty acids	33.4	33.2	33.0	31.8	33.0	31.4	32.3	35.3	32.7
Monounsaturated fatty acids	33.9	42.3	42.5	36.5	40.3	41.7	24.4	36.3	39.3
Polyunsaturated fatty acids	22.6	16.7	16.9	22.4	18.3	16.8	27.7	19.2	19.8
Total lipid content									
(% of dry weight)	23.1	34.6	34.1	24.7	31.9	59.2	18.5	24.5	26.9
Lipid productivity (mg $L^{-1}d^{-1}$)	22	66	44	19	30	50	16	28	20
Biomass productivity (mg $L^{-1}d^{-1}$)	81	173	115	68	84	74	56.	86	66

5.3.12. Cultivation with a two-stage strategy

A two-stage cultivation was used to mass cultivate *Chlorococcum* sp. In the first stage, cells were cultivated in the BG-11 medium (with 1.5 g L^{-1} of $NaNO_3$); in the second stage cells were grown in the modified BG-11 medium (without sodium nitrate) under photoautotrophic and mixotrophic conditions. In the first stage *Chlorococcum* sp. reached a maximum biomass concentration (0.83 ± 0.03 g L^{-1}), total lipid (13 ± 0.3 % of dry weight), lipid mass (0.11 ± 0.0 g L^{-1}) and maximum total fatty acid (8 ± 0.1 % of dry weight). The maximum biomass, total lipid content and fatty acid content are shown in Table 5-10. The maximum lipid mass in the second stage was C1 (0.27 ± 0.03 g L^{-1}), C2 (0.81 ± 0.01 g L^{-1}), C3 (0.57 ± 0.01 mg L^{-1}), C4 (0.30 ± 0.01 mg L^{-1}), C5 (0.34 ± 0.0 mg L^{-1}) and C6 (0.63 ± 0.01 mg L^{-1}). Lipid content increased 2-fold to 8-fold in different media and an increase of fatty acids was also observed. Results showed that a two-stage strategy improved biomass, lipid and fatty acid content in *Chlorococcum* sp. The maximum lipid mass and fatty acid content was achieved in medium supplemented with acetate. No significant effects on biomass content, lipid mass and fatty acid content in media supplemented with pure glycerol (C4) and crude glycerol (C5) were found. Some papers also reported that a two-stage strategy generates high biomass and high lipid content in microalgae cells [154, 169, 173, 272].

Table 5-10. Biomass, lipid and fatty acid content of *Chlorococcum* sp. after nitrogen starvation in the modified BG-11 medium with different carbon sources. C1, modified BG-11 medium; C2 modified BG-11 medium supplemented with acetate 2 g L^{-1}; C3, modified BG-11 medium supplemented with glucose 2 g L^{-1}; C4, modified BG-11 medium supplemented with glycerol 2 g L^{-1}; C5, modified BG-11 medium supplemented with crude glycerol 4 g L^{-1}, C6, modified BG-11 medium supplemented with crude glycerol 20 g L^{-1}. DW is dry weight.

Medium	After nitrate starvation			
	Biomass (DW, g L^{-1})	Total lipid content (% of DW)	Fatty acid content (% of DW)	Lipid productivity (g $L^{-1}d^{-1}$)
C1	1.7 ± 0.05	16 ± 1	9 ± 0	39 ± 3
C2	3.0 ± 0.02	27 ± 1	21 ± 0	116 ± 1
C3	2.6 ± 0.03	22 ± 0	13 ± 1	81 ± 1
C4	1.9 ± 0.02	16 ± 0	17 ± 2	43 ± 1
C5	2.0 ± 0.01	17 ± 1	14 ± 1	49 ± 0
C6	3.0 ± 0.03	21 ± 0	15 ± 0	90 ± 1

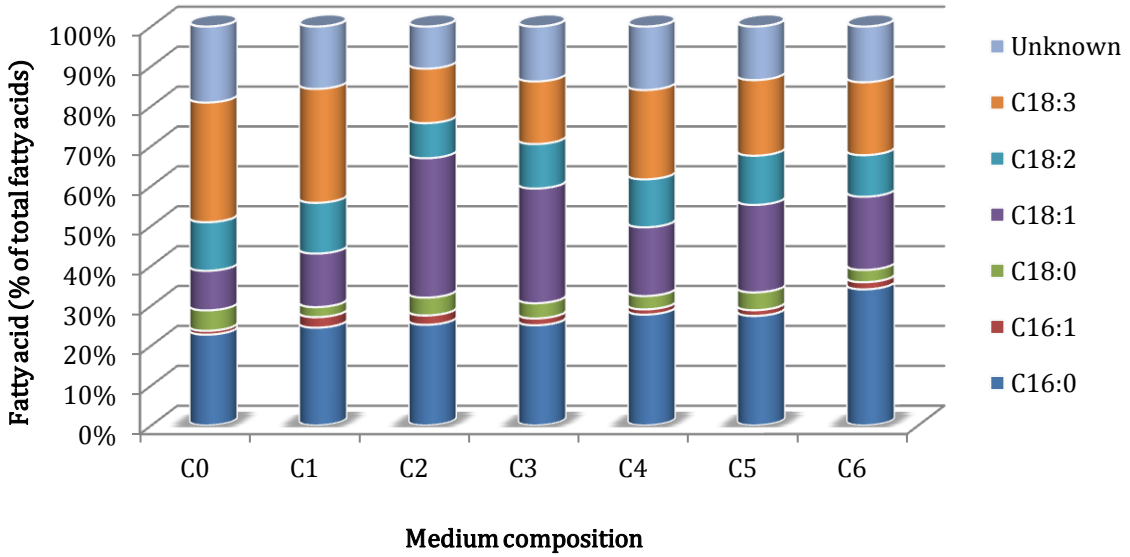


Fig 5-14. Fatty acid composition and profile of *Chlorococcum* sp. C0, BG-11 full medium (no nitrogen starvation); C1, modified BG-11 medium; C2 modified BG-11 medium supplemented with acetate 2 g L^{-1}; C3, modified BG-11 medium supplemented with glucose 2 g L^{-1}, C4, modified BG-11 medium supplemented with glycerol 2 g L^{-1}; C5, modified BG-11 medium supplemented with crude glycerol 4 g L^{-1}, C6, modified BG-11 medium supplemented with crude glycerol 20 g L^{-1}.

Fig. 5-14 shows the fatty acid composition of *Chlorococcum* sp. under a two-stage strategy in different media. *Chlorococcum* sp. produced more polyunsaturated fatty acids (C18:2 and C18:3) under optimal conditions (C0 medium with a high concentration of nitrogen). The percentages of C18:2 and C18:3 were 12% and 30%, respectively, of total fatty acids under favorable conditions. Polyunsaturated fatty acids are necessary for the maintenance of the photosynthetic membrane function [164]. Under nitrate deficiency (C1 to C6 medium) the percentages of C16:0 and C18:1 increased and the percentage of polyunsaturated fatty acids decreased. Nitrogen limitation is the most critical aspect affecting lipid metabolism in algae cells [164]. The highest amount of C18:1 (35% of total fatty acids) is achieved in the modified BG-11 medium supplemented with acetate. *Chlorococcum* sp. has been able to produce C18:3 in the range of 18 % to 22 % of total fatty acids in the modified BG-11 medium supplemented with crude glycerol. These results indicated that the medium composition affects fatty acid composition. Moreover, crude glycerol could be a good carbon source for biomass, lipid and fatty acid production in *Chlorococcum* sp.

5.4. Summary

The effects of light intensity, photoperiod, various nitrogen sources, nitrate concentration, various carbon sources, acetate concentration, salinity, type of salt and utilization of CO_2 on biomass concentration, total lipid content and fatty acid content and composition in *Chlorococcum* sp. were studied. Moreover, two-stage strategy was also evaluated for this strain. The salinity tolerance of *Chlorococcum* sp. was up to 2% (w/v) NaCl. Cells were able to tolerate CO_2 up to 10% (v/v). *Chlorococcum* sp. was able to use a wide variety of nitrogen sources including KNO_3, $NaNO_3$, NH_4NO_3 and yeast extract for growth. Although yeast extract was the best nitrogen source for growth, $NaNO_3$ was selected for the growth of *Chlorococcum* sp. as it is cheaper. The lipid content could be increased to 44% under photoautotrophic conditions. Moreover, cells were able to grow under mixotrophic conditions. The presence of organic carbon, i.e., acetate or glucose as carbon source increased the lipid content in cells. In the presence of acetate (70 mM) in the medium total lipid content up to 56% of dry weight could be increased. A photoperiod did not significantly affect the total lipid content or fatty acid composition; however, culture age and nitrate starvation did. Substitution of NaCl with KCl did not significantly influence lipid content, but the presence of KCl in the medium was more beneficial to accumulating fatty acids. An increasing concentration of carbon dioxide enhanced biomass, lipid and fatty acid productivity. An inhibition of biomass, total lipid and fatty acid production occurred under high light intensity (500 μmol photons $m^{-2}s^{-1}$).

Further, in the two-stage strategy *Chlorococcum* sp. showed its ability to enhance lipid productivity. The highest lipid productivity (116 mg $L^{-1}d^{-1}$) was achieved in the BG-11 medium supplemented with acetate. Cells produced more polyunsaturated fatty acids in the BG-11 medium containing sodium nitrate (1.5 g L^{-1}). Cells in the acetate medium produced more monounsaturated fatty acids (C18:1). However, cells were able to enhance lipid productivity in the modified BG-11 medium supplemented with glycerol and crude glycerol. The lipid productivity achieved was 90 mg $L^{-1}d^{-1}$ in the modified BG-11 medium supplemented with crude glycerol (20 g L^{-1}).

CHAPTER 6
Lipid Production of Marine Microalga *Nannochloropsis* sp.

6.1. Introduction

Nannochloropsis is a genus of green algae. Cells are small, coccoid, non-motile spheres that lack pyrenoids. They are found in marine, brackish and freshwater habitats [1]. Fig 6-1 shows the cells of *Nannochloropsis* sp. used in our experiments. A variety of aquatic microalgae, including the green alga *Nannochloropsis*, were studied for their possible efficacy as bio-resources for fish feed, foods, cosmetics and pharmaceutical products [39].

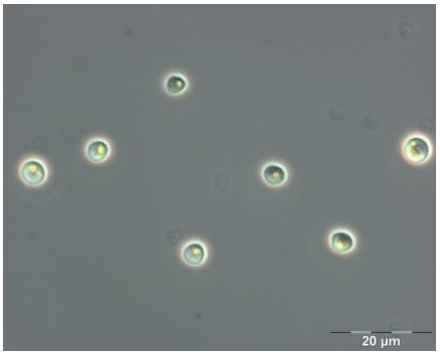


Fig. 6-1. *Nannochloropsis* sp. under light microscope

6.2. Methods

6.2.1. Effect of nitrate concentration

Cells of *Nannochloropsis* sp. were inoculated in f/2 medium with different concentrations of sodium nitrate (25, 75 and 150 mg L^{-1}) to determine the effects of nitrate concentration on growth, lipid content and total fatty acid content. Cells were cultivated under light intensity of 250 µmol photon $m^{-2}s^{-1}$ provided by a white fluorescent lamp on a LD 12:12 h.

6.2.2. Utilization of acetate, crude glycerol and carbon dioxide as a carbon source

Cells were inoculated in f/2 medium with different concentrations of acetate (0, 2, 4, 6 and 8 g L^{-1}) to determine the effects of acetate on biomass, total lipid content and total fatty acid content. The response of growth, lipid content and total fatty acid content

in algae cells was also evaluated in f/2 medium supplemented with crude glycerol (0, 4, 10, 20 and 30 g L^{-1}) and in f/2 medium aerated with CO_2-enriched air (5% v/v). The cultures were cultivated under light intensity of 250 µmol photon $m^{-2}s^{-1}$ provided by a white fluorescent lamp on a LD 12:12 h.

6.3. Results and Discussion

6.3.1. Effect of nitrate concentration on biomass, total lipid content and fatty acid content

To evaluate the effect of nitrate concentration on growth, total lipid content and fatty acid content in a marine microalga, *Nannochloropsis* sp. the f/2 medium containing sodium nitrate from 25 to 150 mg L^{-1} was used. Fig. 6-2 shows the growth curve of *Nannochloropsis* sp. in f/2 medium with different $NaNO_3$ concentrations. As shown in Fig. 6-2, *Nannochloropsis* sp. grew well in a medium containing a high sodium nitrate concentration. The maximum biomass achieved was 0.27, 0.73 and 1.02 g L^{-1} in the f/2 medium containing 25, 75 and 150 mg L^{-1} of $NaNO_3$, respectively (Table 6-1). The lipid content decreased significantly from 28%, 18% and 18% of dry weight; however, the maximum lipid mass obtained was 77, 129 and 180 mg L^{-1} at the end of cultivation.

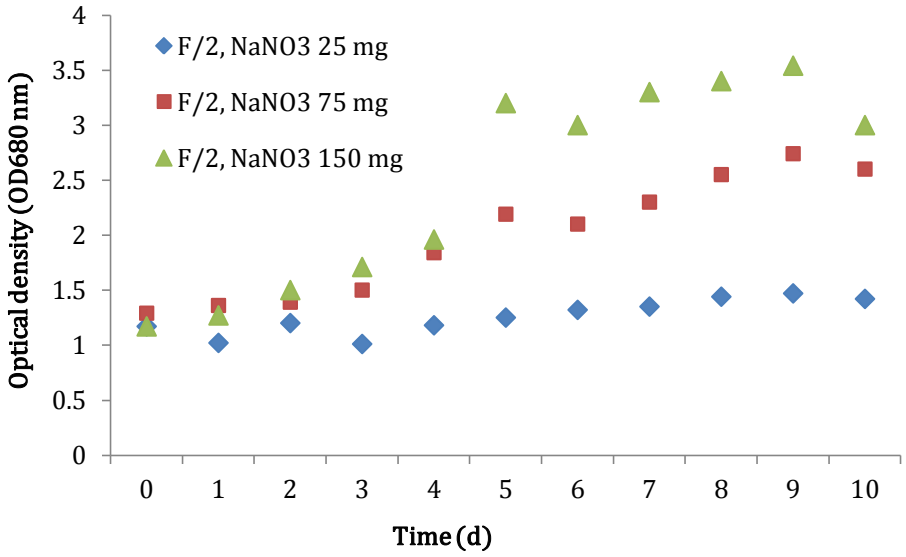


Fig. 6-2. Growth of *Nannochloropsis* sp. in f/2 medium in different concentrations of $NaNO_3$.

A number of algae species are able to accumulate lipids under nitrogen starvation such as *Neochloris oleoabundans* [151], *Chlorella* sp. [150], *Nannochloropsis* sp. [176], *Nannochloropsis oculata* [154], and *Chlorella minutissima* [169]. Our results are in agreement with others in that the nitrogen concentration is a major factor

affecting lipid accumulation in algae cells [110, 150, 151, 155, 176]. At high lipid content the overall lipid and biomass productivity is low [110].

Table 6-1. Summary of fatty acid composition, biomass, total lipid content and total lipid productivity in *Nannochloropsis* sp. at different concentrations of $NaNO_3$.

	F/2 ($NaNO_3$ 25 mg)	F/2 ($NaNO_3$ 75 mg)	F/2 ($NaNO_3$ 150 mg)
Fatty acid composition (%):			
C16:0	31.2 ± 0.8	32.0 ± 0.2	29.4 ± 0.0
C16:1	2.2 ± 0.1	2.3 ± 0.1	2.1 ± 0.0
C18:0	5.5 ± 0.2	5.3 ± 0.0	5.4 ± 0.1
C18:1	19.7 ± 0.4	20.2 ± 0.2	19.2 ± 0.2
C18:2	20.1 ± 0.2	19.9 ± 0.3	19.4 ± 0.4
C18:3	9.0 ± 0.2	8.3 ± 0.1	8.2 ± 0.1
Unknown fatty acids	12.4 ± 0.9	12.0 ± 0.1	16.3 ± 0.3
Saturated fatty acids	36.7	37.3	34.8
Unsaturated fatty acids	51	50.7	48.9
Biomass (g L^{-1})	0.27 ± 0.01	0.73 ± 0.04	1.02 ± 0.08
Total lipid content (% of dry weight)	29 ± 2	18 ± 1	18 ± 1.7
Lipid productivity (mg $L^{-1}d^{-1}$)	8 ± 0.1	13 ± 1	18 ± 2

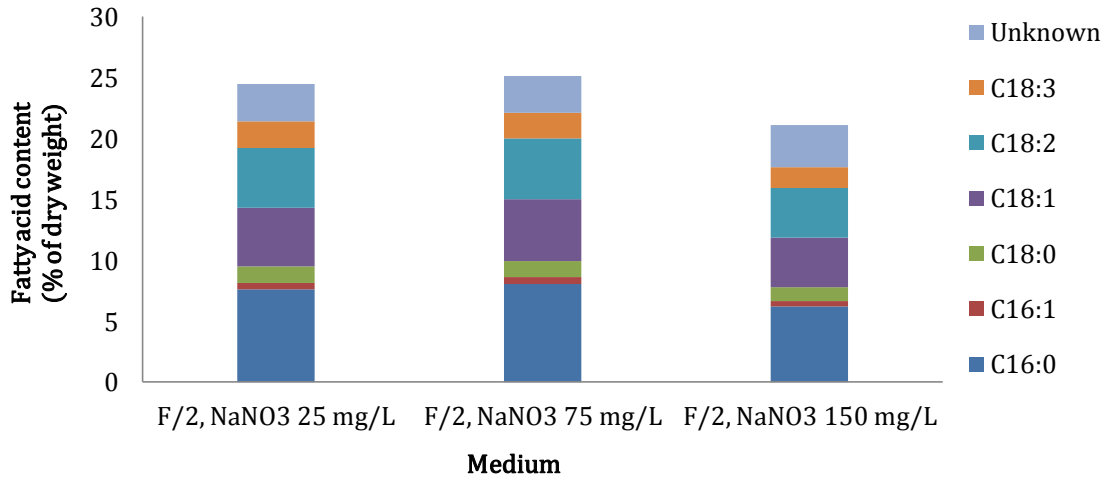


Fig. 6-3. Fatty acid content and profile of *Nannochloropsis* sp. at different concentrations of $NaNO_3$.

Fig. 6-3 shows the fatty acid content and profile of *Nannochloropsis* sp. at different levels of $NaNO_3$. The reduction of $NaNO_3$ concentration in the medium increased the fatty acid content. The maximum fatty acid concentration achieved was 66, 183 and 215 mg L^{-1} at the end of cultivation in the f/2 medium containing 25, 75 and 150 mg L^{-1} of $NaNO_3$, respectively. Overall, the compositions of fatty acid were similar at different levels of $NaNO_3$, with 35-37% saturated fatty acids, 49-51% unsaturated fatty acids and 12-16% unknown fatty acids. The most abundant saturated fatty acid was palmitic acid (C16:0) and the most abundant unsaturated fatty acids were oleic acid (C18:1) and linoleic acid (C18:2) (Table 6-1).

6.3.2. Utilization of acetate, crude glycerol and carbon dioxide as carbon source

To investigate the effect of carbon source on lipid content in cells of *Nannochloropsis* sp., the modified f/2 medium containing different carbon sources including acetate, glucose, glycerol, crude glycerol and carbon dioxide was used. The lipid accumulation of cells in different media was assessed with Nile red dye using a fluorescence microscope; the lipid droplets displayed a yellow color (Fig. 6-4).

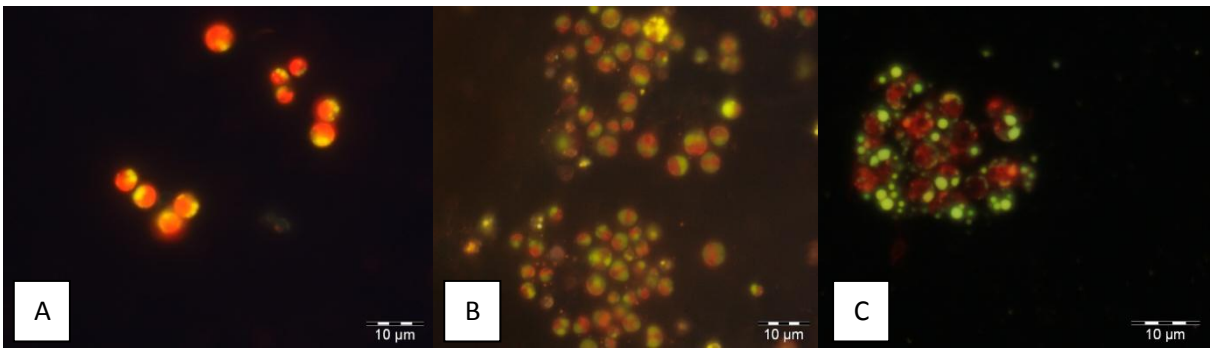


Fig. 6-4. *Nannochloropsis* sp. cells under fluorescence microscope (A) in f/2 medium, (B) f/2 medium with acetate and (C) f/2 medium with crude glycerol.

The time course profiles of cell growth obtained in the modified f/2 medium are shown in Fig. 6-5A. As shown in Fig. 6-5A the growth of *Nannochloropsis* sp. improved significantly when cells were grown in a medium containing carbon sources including organic carbon (acetate, glycerol and crude glycerol) and inorganic carbon (CO_2-enriched air). Fig 6-5B presents the biomass concentration (as a dry weight) and biomass productivity of *Nannochloropsis* sp. in different media. In the presence of acetate (8 g L^{-1}) the biomass productivity increased.

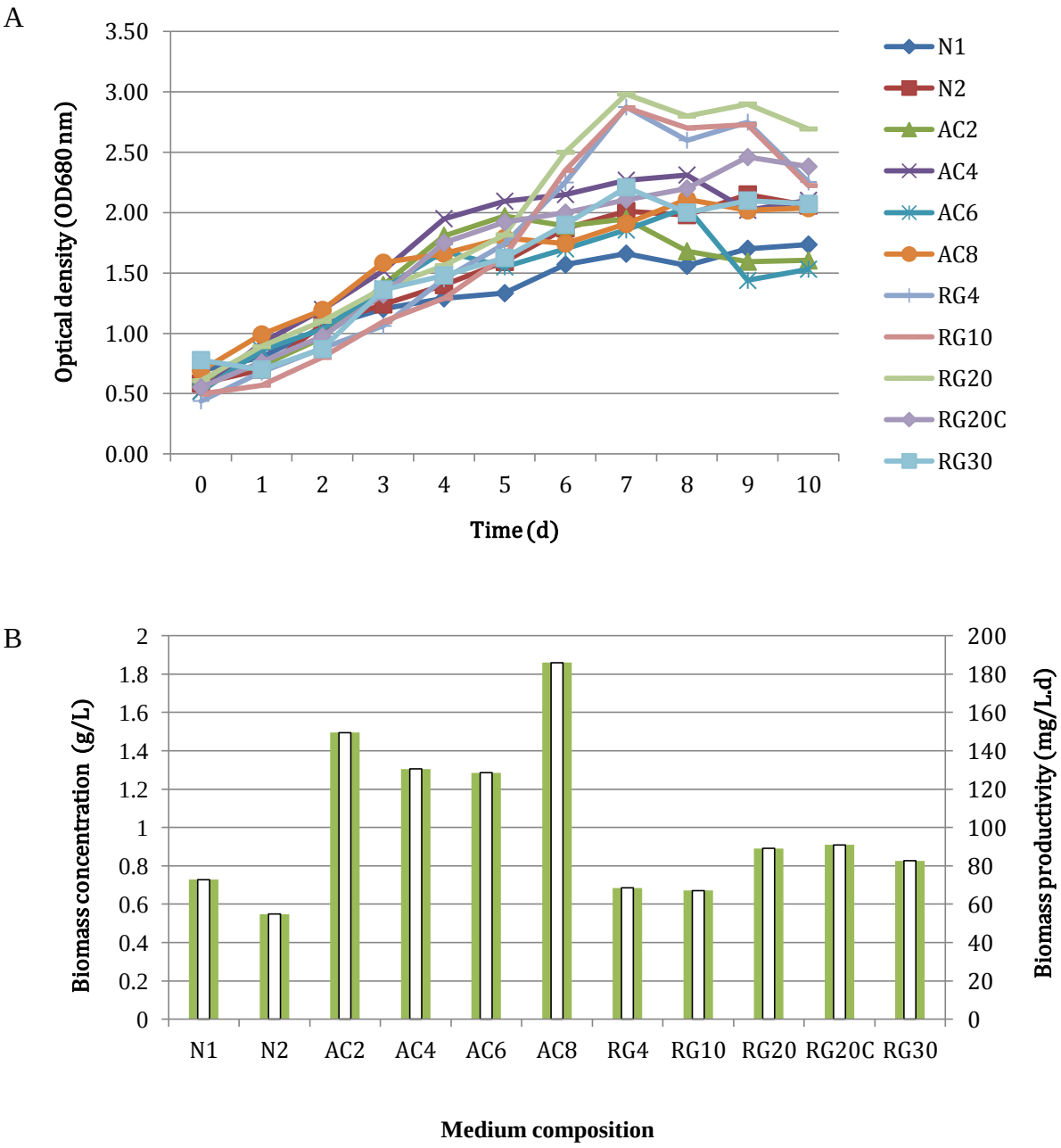


Fig. 6-5. (A) Growth of *Nannochloropsis* sp. and (B) biomass concentration and biomass productivity in different media. N1, f/2 medium; N2, f/2 medium with CO_2-enriched air; AC2, f/2 medium supplemented with acetate 2 g L^{-1}, AC4, f/2 medium supplemented with acetate 4 g L^{-1}; AC6, f/2 medium supplemented with acetate 6 g L^{-1}; AC8, f/2 medium supplemented with acetate 8 g L^{-1}; RG4, f/2 medium supplemented with crude glycerol 4 g L^{-1}; RG10, f/2 medium supplemented with crude glycerol 10 g L^{-1}, RG20, f/2 medium supplemented with raw glycerol 20 g L^{-1}; RG20C, f/2 medium supplemented with crude glycerol 20 g L^{-1} and aerated with CO_2-enriched air; RG30, f/2 medium supplemented with crude glycerol 30 g L^{-1}. Filled and unfilled columns represent biomass concentration and biomass productivity, respectively.

Fig. 6-6 shows total lipid content and total fatty acid content obtained in the modified f/2 medium. When the cultures were aerated with CO_2-enriched air (5% v/v) a downward shift of pH of about 0.32-1.35 was observed, and the total lipid productivity was 2.5-fold higher than under air aeration. The acetate uptake was about 27% in the f/2 medium supplemented with 2 g L^{-1} acetate and the cell uptake of acetate was very low in other mediums. In the f/2 medium supplemented with crude glycerol cells produced glycerol. In the presence of acetate total lipid content increased slightly. Besides that, the growth of cells in acetate media was observed in a broad range of pH from 7.92 to 8.28. The lipid content increased significantly when the microalgae cells were grown under mixotrophic conditions with crude glycerol as a carbon source. Moreover, the highest lipid was obtained in f/2 medium supplemented with crude glycerol (20 g L^{-1}) and aerated with CO_2 (5% v/v CO_2 in air). As shown in Fig 6-6B, the maximum fatty acid content achieved was 39% of dry weight under mixotrophic conditions with crude glycerol as a carbon source and aerated with CO_2 (5% v/v CO_2 in air). Crude glycerol was a better candidate than acetate as an organic carbon source to enhance lipid content in *Nannochloropsis* sp. Acetate, glucose, glycerol, and sucrose are the most frequently used sources of carbon. Organic carbons are able to trigger a lipid accumulation in algae cells, whereby the carbon source cannot be used for the protein synthesis, but can be used for lipid synthesis [173].

Table 6-3 shows that the cells produced more unsaturated fatty acids (60% of total fatty acids) with air aeration or CO_2-enriched air. In addition, cells also produced more unsaturated fatty acids (53-57%) in the medium supplemented with acetate. When cells were grown in the medium supplemented with glycerol, more saturated fatty acids (48-52% of total fatty acids) were produced. The fatty acid production under photoautothrophic and mixotrophic conditions was investigated in further experiments. The selected media were f/2 medium, f/2 medium aeration with CO_2 (5% v/v CO_2 in air), f/2 medium supplemented with acetate (8 g L^{-1}), f/2 medium supplemented with acetate (8 g L^{-1}) and aerated with CO_2 (5% v/v CO_2 in air), f/2 medium supplemented with crude glycerol (20 g L^{-1}) and f/2 medium containing crude glycerol (20 g L^{-1}) and aerated with CO_2 (5% v/v CO_2 in air).

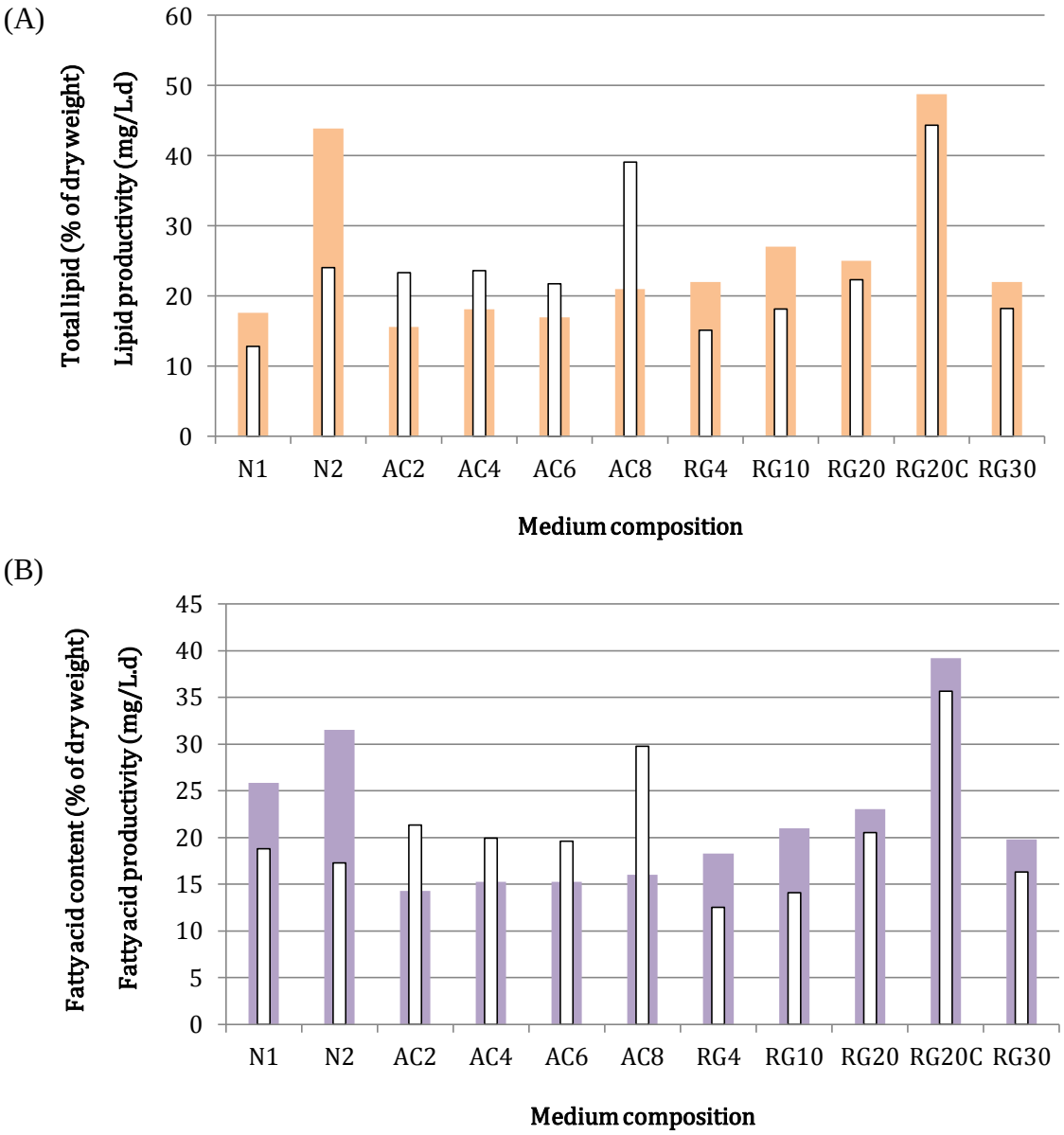


Fig. 6-6 (A) total lipid content and lipid productivity and (B) fatty acid content and productivity of *Nannochloropsis* sp. in different media. N1, f/2 medium; N2, f/2 medium with CO_2-enriched air; AC2, f/2 medium supplemented with acetate 2 g L^{-1}, AC4, f/2 medium supplemented with acetate 4 g L^{-1}; AC6, f/2 medium supplemented with acetate 6 g L^{-1}; AC8, f/2 medium supplemented with acetate 8 g L^{-1}; RG4, f/2 medium supplemented with crude glycerol 4 g L^{-1}; RG10, f/2 medium supplemented with crude glycerol 10 g L^{-1}, RG20, f/2 medium supplemented with crude glycerol 20 g L^{-1}; RG20C, f/2 medium supplemented with crude glycerol 20 g L^{-1} and aerated with CO_2-enriched air; RG30, f/2 medium supplemented with crude glycerol 30 g L^{-1}. Filled columns represent total lipid and fatty acid content. Unfilled columns represent lipid and fatty acid productivity.

Table 6-3. Summary of fatty acid composition of *Nannochloropsis* sp. in different media at the end of cultivation. N1, f/2 medium; N2, f/2 medium with CO_2-enriched air; AC2, f/2 medium supplemented with acetate 2 g L^{-1}, AC4, f/2 medium supplemented with acetate 4 g L^{-1}; AC6, f/2 medium supplemented with acetate 6 g L^{-1}; AC8, f/2 medium supplemented with acetate 8 g L^{-1}; RG4, f/2 medium supplemented with crude glycerol 4 g L^{-1}; RG10, f/2 medium supplemented with crude glycerol 10 g L^{-1}, RG20, f/2 medium supplemented with crude glycerol 20 g L^{-1}; RG20C, f/2 medium supplemented with crude glycerol 20 g L^{-1} and aerated with CO_2-enriched air; RG30, f/2 medium supplemented with crude glycerol 30 g L^{-1}.

Fatty acid (%)	N1	N2	AC2	AC4	AC6	AC8	RG4	RG10	RG20	RG20C	RG30
C16:0	32.1	29.2	28.1	28.8	27.5	28.5	38.5	42.8	45.8	38.4	46.1
C16:1	2.1	2.8	1.6	1.7	1.6	1.8	2.3	2.2	2.3	3.3	3.1
C18:0	6.6	5.5	10.1	9.9	10.2	10.3	5.8	5.2	4.9	4.4	5.7
C18:1	20.2	32.6	30.1	29.8	30.2	30.4	19.3	13.8	13.3	21.9	11.6
C18:2	20.2	16.7	15.8	15.7	15.8	18.2	18.0	16.1	15.7	17.1	16.5
C18:3	8.5	5.8	5.2	5.6	5.2	5.0	9.0	10.1	9.7	6.5	7.6
Unknown fatty acids	10.3	7.4	9.2	8.5	9.5	5.8	7.1	9.8	8.2	8.4	9.5
Saturated fatty acids	38.7	34.7	38.2	38.7	37.5	38.8	36.3	48	50.7	42.8	51.8
Unsaturated fatty acids	60	57.9	52.6	52.8	53	55.4	56.6	42.2	41.1	48.8	38.7

Figs. 6-8 and 6-9 show the time course of fatty acid content and its profile, and fatty acid composition, respectively. Results show that fatty acid content increased with increasing culture time. Biosynthesis of fatty acids was faster when cells were grown in f/2 medium aerated with CO_2 (Fig. 6-8). The maximum fatty acid content achieved 27% of dry weight after 7 days of cultivation in f/2 medium aerated with CO_2 (Fig. 6-8B), whereas a fatty acid content of 26% was reached in f/2 medium aerated with air (Fig. 6-8A). Increasing culture time and increasing C16:0 and C18:1 was observed. Palmitic acid (C16:0) increased from 23% to 32% of total fatty acids and oleic acid (C18:1) increased from 11% to 22% in f/2 medium aerated by air (Fig. 6-9A). A significant increase was obtained for palmitic acid from 23% to 40% in f/2 medium aerated by carbon dioxide (Fig. 6-9B). A decrease of three fatty acids (C18:0, C18:2 and C18:3) and unknown fatty acids in both media was observed. As shown in Fig, 6-9C, palmitic acid, stearic acid and oleic acid increased from 23% to 31%, 6% to 9% and 11% to 25%, respectively, when cells were cultivated in f/2 medium supplemented with acetate and aerated with air. Linoleic acid (C18:2) linolenic acid (C18:3) and unknown fatty acids decreased from 25% to 20%, 11% to 7% and 21% to 7%, respectively. The growth of *Nannochloropsis* sp. stopped after 5 days of cultivation when the cells were grown in f/2 medium supplemented with acetate and aerated by CO_2. The pH of culture was from 5 to 6. As a way of explanation, in an acidic lake with a pH below 5, CO_2 exists only as inorganic carbon, which limits the photosynthesis process and the growth of phytoplankton if other nutrients are relatively abundant [273]. In addition, Heifetz at *al*. [274] reported that the growth of *Clamydomonas reinhardtii* in increasing concentrations of acetate with CO_2 (5% v/v) aeration in liquid culture progressively reduced photosynthetic CO_2 fixation and net O_2 evolution without effects on respiration, photosystem II efficiency (as measured by chlorophyll fluorescence) or growth.

Results show that palmitic acid increased significantly from 23% to 46% in f/2 medium supplemented with crude glycerol and aerated with air (Fig. 6-9D). Under the same conditions an increase of C18:1 (from 11% to13%) occurred, whereas C18:2, C18:3 and unknown fatty acids decreased from 25% to 16%, 11% to 10% and 21% to 8%, respectively. An increase of linolenic acid up to 23% was observed after 3 days of incubation. The maximum fatty acid production with *Nannochloropsis* sp. was achieved in f/2 medium supplemented with crude glycerol and aerated by CO_2 (Fig. 6-9E). Palmitic acid and oleic acid increased from 23% to 38% and 11% to 22%, respectively and C18:2, C18:3 and unknown fatty acids decreased from 25% to 17%, 11% to7% and

21% to 8%, respectively. Overall, the results show that fatty acid composition varied depending on the medium composition and the age of culture. Content of C16:0 and C18:1 increased with increasing culture time and became predominantly fatty acid at the end of cultivation. Otherwise, the percentage of palmitoleic acid (C16:1), linoleic (C18:2), linolenic (C18:3) and unknown fatty acids decreased with culture time. The composition of fatty acids was influenced by the amount of nitrate in the media. Further, after 3 days of cultivation nitrate was not observed. An increase of C18:1 under nitrate starvation was also reported in *Nannochloropsis* sp. [176], *Chlorella minutissima* UTEX 2341 [169] and *Nannochloropsis oculata* [154]. Nitrate starvation has a tendency to accumulate C16:0 and C18:1. Then a high composition of oleic acid (C18:1) was followed by a low composition of C18:2 and C18:3 [169, 176]. During logarithmic growth, most lipids are glycerol-based polar membrane lipids which function to maintain cell structure and TAG production, which is enhanced under stress conditions [164]. Hu et *al*. [164] reported that an increase in the relative proportions of saturated and monounsaturated fatty acids (i.e. C16:0 and C18:1) and a decrease in the proportion of polyunsaturated fatty acids in total lipid were also associated with growth-phase transition from the logarithmic to the stationary phase.

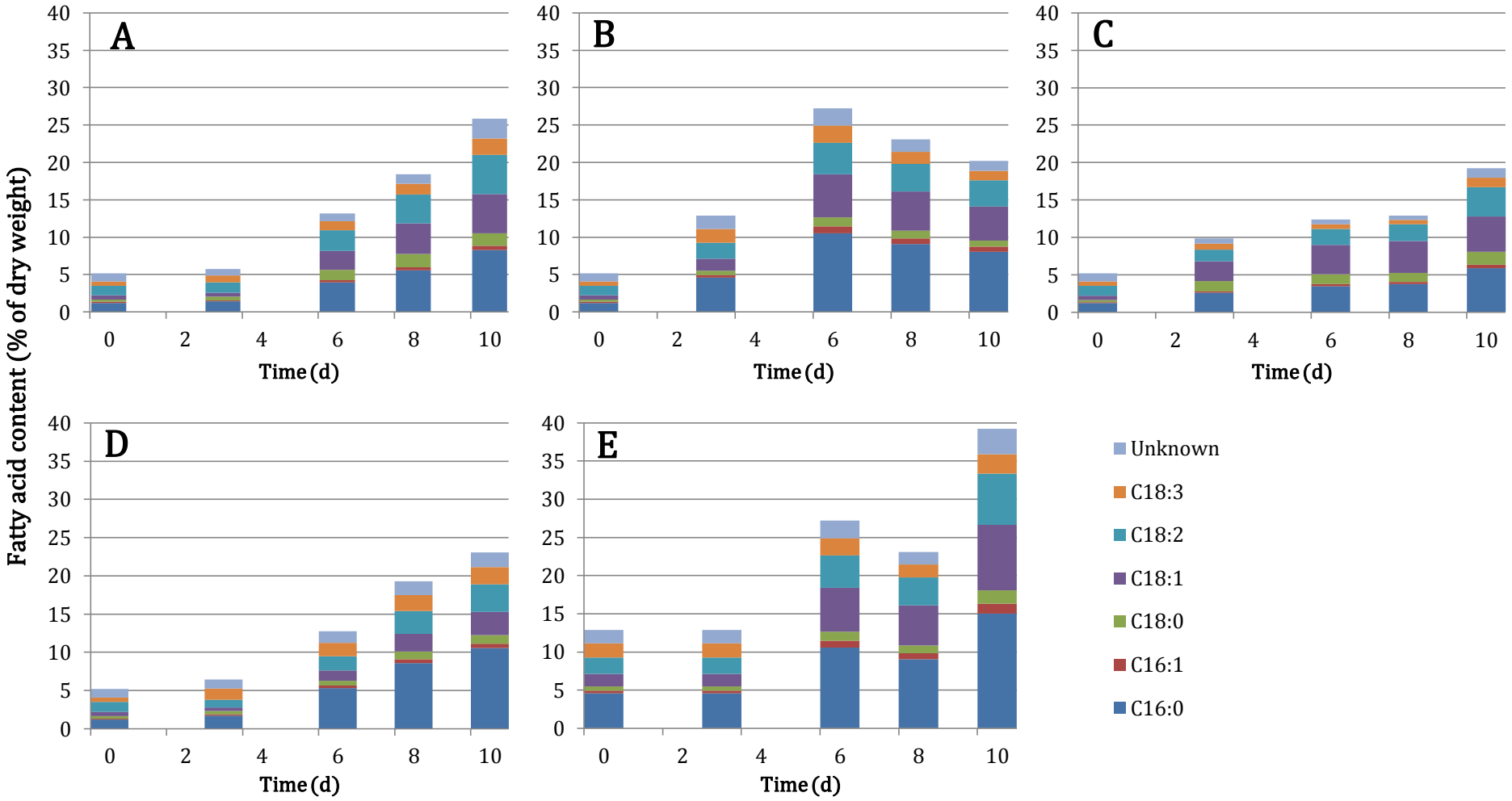


Fig. 6-8. Time course of fatty acid content and its profile of *Nannochloropsis* sp. in f/2 medium (A), f/2 medium aerated with CO_2-enriched air (B), f/2 medium containing acetate 8 g L^{-1} (C), f/2 medium containing crude glycerol 20 g L^{-1} (D) and f/2 medium containing crude glycerol 20 g L^{-1} and aerated with CO_2-enriched air (E).

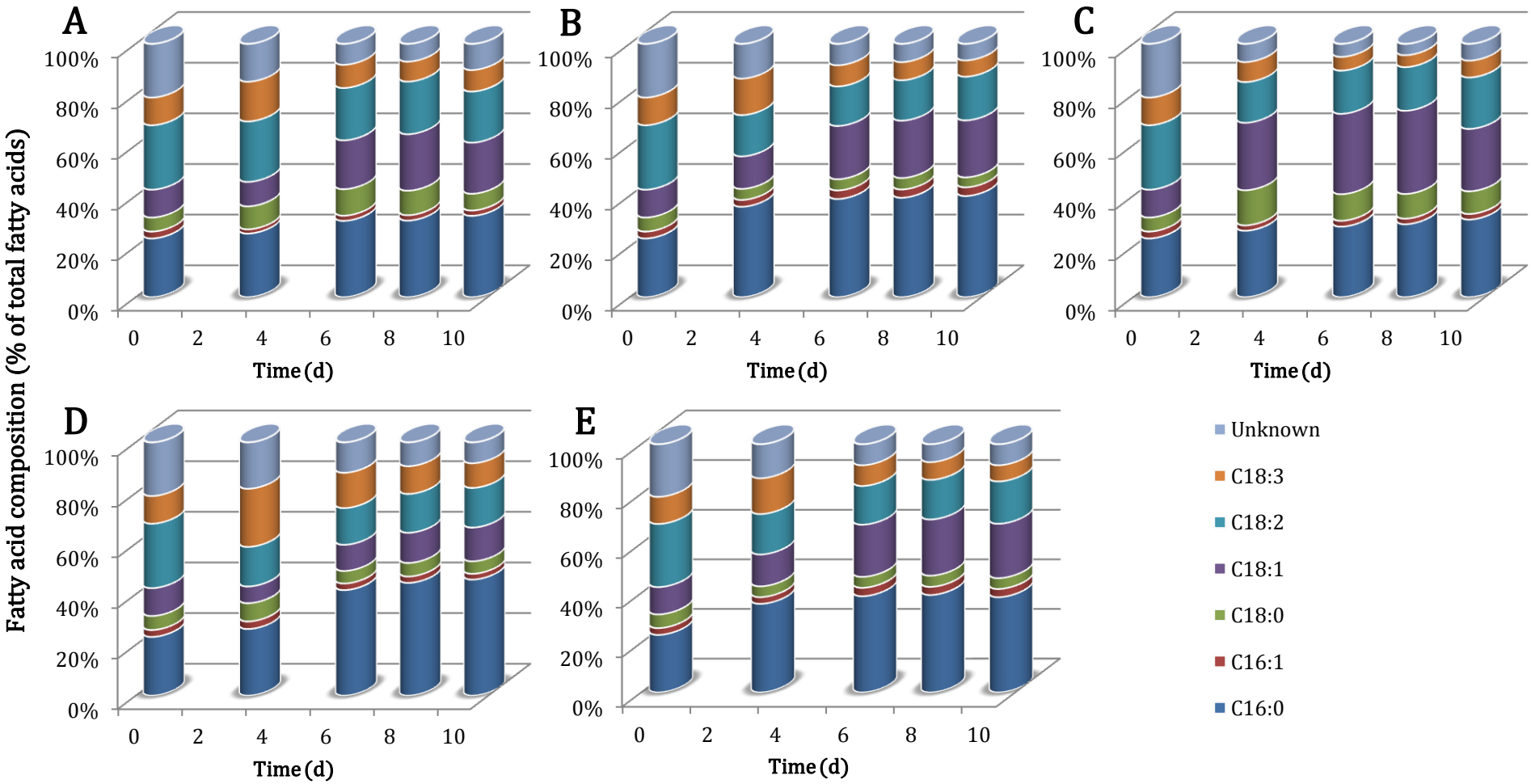


Fig. 6-9. Time course of fatty acid composition of *Nannochloropsis* sp. in f/2 medium (A), f/2 medium aerated with CO_2-enriched air (B), f/2 medium containing acetate 8 g L^{-1} (C), f/2 medium containing crude glycerol 20 g L^{-1} (D) and f/2 medium containing crude glycerol 20 g L^{-1} and aerated with CO_2-enriched air (E).

6.4. Summary

Some strategies for enhancing lipid productivity with *Nannochloropsis* sp. such as different levels of sodium nitrate and various carbon sources like acetate and crude glycerol were used. *Nannochloropsis* sp. showed high growth in medium with a high sodium nitrate concentration. The highest lipid mass achieved was 180 mg L^{-1} during 10 days of cultivation in the f/2 medium containing 150 mg L^{-1} sodium nitrate, representing 18% (dry weight) of total lipid content. The reduction of $NaNO_3$ concentration in the medium increased the fatty acid content. The composition of fatty acids were similar at different levels of $NaNO_3$, with 35-37% saturated fatty acids, 49-51% unsaturated fatty acids and 12-16% unknown fatty acids. The most abundant saturated fatty acid was palmitic acid and the most abundant unsaturated fatty acids were oleic acid and linoleic acid.

Nannochloropsis sp. was able to grow under mixotrophic conditions. The presence of organic carbon (acetate or crude glycerol) as carbon source in the medium increased the lipid content in cells. The combination of crude glycerol (20 g L^{-1}) and carbon dioxide (5%, v/v) was a good candidate for increasing the lipid content in *Nannochloropsis* sp. Under these conditions cells accumulated lipids up to 48% of dry weight. Under photoautotrophic and under mixotrophic conditions in the presence of acetate, cells produced more unsaturated fatty acids, whereas cells produced more saturated fatty acids under mixotrophic conditions with glycerol as carbon source.

CHAPTER 7

Evaluation of Antioxidant Potential in *Chlorococcum* sp. and *Nannochloropsis* sp.

7.1. Introduction

An antioxidant is a substance that, when present in low concentrations, compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substance [275]. The antioxidant compounds play a vital role in preventing cancer, heart disease, and neurodegenerative diseases and the aging process [276, 277]. Antioxidants are found in a wide variety of products including food, cosmeceuticals and drugs. Antioxidants are added to a variety of foods to prevent oxidative processes and help maintain the flavor, texture and color of food during storage [278, 279] due to the formation of free radicals caused by environmental factors such as air, light and temperature. In cosmeticeutical products, the antioxidant compounds are designed to improve the appearance of the skin [280]. Antioxidants applications are presently on the market which prevent the aging process and UV-induced skin damage, as well as the threat of wrinkles and erythema due to inflammation [281]. Mostly synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ), propyl gallate (PG), are commonly used in food, food supplements as well as cosmetic formulations. Because of their toxicity and potential carcinogenicity, research is being carried out to replace them with products from plants or microorganisms.

Carotenoids are important antioxidant compounds from microalgae. They can be divided into two groups: carotenes and xanthophylls. Carotenoids have the ability to quench reactive oxygen species (ROS) especially singlet molecular oxygen ($^1O_2$) and trap peroxyl radicals [68]. ROS attack molecules such as lipids, proteins, carbohydrates, as well as nucleic acids that cause cell or tissue injury associated with aging, atherosclerosis, carcinogenesis and heart disease [282, 283]. The presence of carotenoids in microalgae and a correlation between carotenoid content and antioxidant capacity and were reported [284-286].

Other important antioxidants are phenolic compounds, which can be divided into two groups: flavonoids (polyphenolic compounds) and non-flavonoids (phenolic acids,

ellagic acid, gallic acid, hydroxycinnamates, etc). Many flavonoids were shown to have antioxidative activity, free-radical scavenging capacity, coronary heart disease prevention, and anticancer activity, while some flavonoids exhibit potential for anti-human immunodeficiency virus functions [287]. Flavonoids can suppress free radical formation either by chelating trace metals or inhibiting enzymes and scavenging free radicals including superoxide anion (O_2•-), peroxyl (ROO•), alkoxyl (RO•), and hydroxyl (HO•) [288]. Until now, the presence of phenolic compounds in microalgae and a correlation between antioxidant capacity and phenolic content has been rarely reported [75, 289-291].

Microalgae are abundant in nature and can be used as a renewable source of natural antioxidants. A number of microalgae have been used in the commercial production of pigments with antioxidant properties, for example: astaxanthin from *Haematococcus pluvialis*, ß-carotene from *Dunaliella salina*, as well as phycobiliproteins from *Arthrosphira* and *Phorphyridium* [39].

7.2. Materials and methods

7.2.1. Biomass

Biomass was obtained from previous experiments. *Chlorococcum* sp. was cultured in BG-11 medium at pH 8 with different concentrations of acetate (0-12 g L^{-1}), sodium chloride (0-1% w/v), potassium chloride (0-1% w/v), aeration with 6% (v/v) CO_2 (v/v) as well as under different light intensities, 100 µmol photons $m^{-2}s^{-1}$ for Batch 1 and 4, 250 µmol photons $m^{-2}s^{-1}$ for Batch 2, and 500 µmol photons $m^{-2}s^{-1}$ for Batch 3. *Nannochloropsis* sp. was cultivated in f/2 medium at pH 8 with containing sodium nitrate (25-150 mg L^{-1}), acetate (0-8 g L^{-1}), crude glycerol (0-30 g L^{-1}) as well as aeration with 5% (v/v) CO_2.

7.2.2. Extraction

Extraction was carried out in an ethanol/water mixture according to Goiris et *al*. [289]. Briefly, 100 mg of biomass powder and 2 mL of ethanol-water (3:1, v/v) were mixed and stirred for 30 minutes. After the process was completed, the samples were centrifuged at 4500 rpm for 10 minutes. The residue was then extracted for a second time in ethanol-water. Sample solutions were polled and stored at -20°C.

7.2.3. Determination of total phenolic content and total carotenoid content

The phenolic content was determined by the Folin-Ciocalteau test according to Al-Duais et *al*. [292]. An amount of 20 µl extract was mixed with 100 µl of Folin-Ciocalteau reagent from Sigma (diluted tenfold with distilled water before use) and allowed to stand for 5 minutes at room temperature. 75 µl of 60 g L^{-1} sodium bicarbonate solution was added and mixed well. The mixture was incubated for 120 minutes in the dark at room temperature. The absorbance was measured at 740 nm. Gallic acid was prepared from 0 to 150 µg mL^{-1} in water as standard solution. The phenolic content was expressed as mg Gallic acid equivalent (GAE) per 100 g of the dry weight.

The total carotenoid content was measured using a spectrophotometer according to Lichtenthaler and Busmann [293]. The extracts were diluted (5-50 times) with 90% (v/v) methanol in water and absorbance was measured at 470, 652 and 665 nm. Lichtenthaler equations were used to calculate the total carotenoid content.

7.2.4. DPPH assay

A microplate reader and 96-well plates were used to determine the scavenging activity with 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals. This assay is based on the method described by Mensor et *al*. [294]. In this method, 50 µL of a daily prepared ethanol DPPH solution (0.3 mM) was mixed with 200 µL of extract solution and allowed to react at room temperature in the dark. After 1 hour, the absorbance was measured at 517 nm. The mixture of DPPH solution (50 µL) and ethanol (200 µL) was used as negative control. Ascorbic acid was used as positive control, at the same concentrations of extract (0, 2, 5, 10, 15 and 20 µg mL^{-1}). A blank solution was the mixture 250 µL of ethanol and sample extract. Results were converted into percentage antioxidant activity (AA) using the following equation:

$$AA\% = 100 - \{[(A_{sample} - A_{blank}) \text{ X } 100] / A_{control}\} \qquad (1)$$

The values of AA% obtained from Eq. 1 were plotted against the ascorbic acid standard concentration. A linear regression ($R^2 = 0.989$) was used to calculate the radical scavenging capacity of the samples (expressed as mg ascorbic acid equivalents (AAE)/100 g sample).

7.2.5. Statistical analysis

All the results were calculated as the mean value ± standard deviation (SD). The analysis of the correlation of antioxidant capacity, total phenolic and carotenoid content used a multiple regressions analysis in Microsoft Excel.

7.3. Results and discussion

7.3.1. Total phenolic content, total carotenoid content and antioxidant capacity

Total phenolic content was estimated using the Folin-Ciocalteau reagent. Total phenolic content is expressed as a Gallic acid equivalent (GAE). Table 7-1 shows the total phenolic content of *Chlorococcum* sp. and *Nannochloropsis* sp. under photoautotrophic and mixotrophic conditions. The results show that *Chlorococcum* sp. had the highest total phenolic content with 241 mg GAE/100 g dry weight in the modified BG-11 medium supplemented with KCl (1% w/v), whereas *Nannochloropsis* sp. had the highest total phenolic content with 205 mg GAE/100 g dry weigh in the f/2 medium with a concentration of 150 mg L^{-1} of sodium nitrate. In the present study, the medium composition had a significant influence on to the total phenol content of *Chlorococcum* sp. and *Nannochloropsis* sp. Fruits like grapes, apples, pears, cherries and berries contain 200–300 mg polyphenols per 100 grams of fresh weight [295].

Carotenoid content was determined spectrophotometrically according to Lichtenhaler and Buschmann's method [293]. The results show that the highest content of carotenoid were 76 and 86 mg/100 g of dry weight in *Chlorococcum* sp. and *Nannochloropsis* sp., respectively (Table 7-1 and 7-2). When the cells were grown in the medium supplemented with acetate or crude glycerol, the carotenoid content was lower than in the medium without organic carbon. The carotenoid content in both species was influenced by nutrient availability and light intensity. Yellow, orange and red carotenoids have industrial use in food products and cosmetics as vitamin supplements and health food products, and as feed additives for poultry, livestock, fish, and crustaceans [296].

Table 7-1. Total phenolic content, total carotenoid content, DPPH analysis, and antioxidant capacity of *Chlorococcum* sp. in different media.

Medium	Total phenolic content (mg GAE/100 g of DW)	Total carotenoid content (mg/100 g of DW)	DPPH (% AA)	Antioxidant capacity (mg AAE/100 g of DW)
Batch 1				
BG-11	80 ± 6	26 ± 0.6	35 ± 2	44 ± 1.2
BG-11 + Acetate 70 mM	46 ± 1	28 ± 0.2	31 ± 2	39 ± 0.8
BG-11+ Acetate 70 mM + NaCl 1%	58 ± 2	32 ± 0.7	39 ± 3	44 ± 0.5
Batch 2				
BG-11	68 ± 0	26 ± 0.6	34 ± 1	44 ± 0.2
BG-11 (with $NaNO_3$ 1.5 g L^{-1})	89 ± 1	36 ± 0.9	42 ± 3	46 ± 0.6
BG-11 + CO_2 6%	96 ± 7	10 ± 0.1	31 ± 2	39 ± 0.4
BG-11 + NaCl 1%	232 ± 8	76 ± 0.5	64 ± 2	60 ± 1.4
BG-11+ KCl 1%	241 ± 7	76 ± 0.4	73 ± 4	65 ± 0.8
BG-11+ Acetate 70 mM	43 ± 3	27 ± 0.1	40 ± 2	44 ± 0.3
BG-11+ Acetate 70 mM + NaCl 1%	173 ± 8	24 ± 0.7	50 ± 2	51 ± 0.4
BG-11 + Acetate 70 mM + KCl 1%	192 ± 7	60 ± 0.5	46 ± 4	48 ± 0.9
Batch 3				
BG-11	61 ± 3	21 ± 0.3	31 ± 2	39 ± 1.3
BG-11 + Acetate 70 mM	45 ± 3	27 ± 0.2	18 ± 2	31 ± 0.4
BG-11+ Acetate 70 mM + NaCl 1%	125 ± 5	21 ± 0.8	47 ± 5	49 ± 1.1
Batch 4				
BG-11	109 ± 1	39 ± 0.0	23 ± 4	34 ± 0.8
BG-11+ Acetate 7 mM	117 ± 0	23 ± 0.4	18 ± 3	31 ± 0.6
BG-11+ Acetate 70 mM	82 ± 2	20 ± 0.6	18 ± 3	31 ± 0.5
BG-11+ Acetate 140 mM	79 ± 3	20 ± 0.9	12 ± 3	27 ± 0.2
BG-11+ Acetate 210 mM	91 ± 3	25 ± 0.3	12 ± 3	27 ± 0.6
BG-11+ Acetate 280 mM	122 ± 3	23 ± 0.0	17 ± 2	30 ± 0.4

Table 7-2. Total phenolic content, total carotenoid content, DPPH analysis and antioxidant capacity of *Nannochloropsis* sp. in different media.

Medium	Total phenolic content (mg GAE/100 g of DW)	Total carotenoid content (mg/100 g of DW)	DPPH (% AA)	Antioxidant capacity (mg AAE/100 g of DW)
F/2[a]	85 ± 3	17 ± 1	11 ± 3	26 ± 1.2
F/2[b]	119 ± 9	86 ± 2	61 ± 2	58 ± 0.4
F/2[c]	205 ± 5	56 ± 2	14 ± 0	35 ± 0.2
F/2[b,d]	49 ± 1	20 ± 1	26 ± 1	36 ± 0.2
F/2[b] + Acetate 2 g L^{-1}	57 ± 0	29 ± 0	8 ± 2	24 ± 1.6
F/2[b] + Acetate 4 g L^{-1}	53 ± 1	26 ± 1	6 ± 1	23 ± 1.3
F/2[b] + Acetate 6 g L^{-1}	60 ± 3	24 ± 0	11 ± 0	26 ± 0.1
F/2[b] + Acetate 8 g L^{-1}	49 ± 2	41 ± 2	16 ± 1	30 ± 0.3
F/2[b] + Crude glycerol 4 g L^{-1}	102 ± 2	60 ± 0	29 ± 5	37 ± 1.0
F/2[b] + Crude glycerol 10 g L^{-1}	98 ± 2	53 ± 1	25 ± 1	35 ± 2.0
F/2[b] + Crude glycerol 20 g L^{-1}	85 ± 6	54 ± 1	29 ± 0	37 ± 0.1
F/2[b,d] + Crude glycerol 20 g L^{-1}	92 ± 4	27 ± 0	27 ± 3	36 ± 0.6
F/2[b] + Crude glycerol 30 g L^{-1}	87 ± 6	53 ± 2	28 ± 1	37 ± 0.2

DPPH analysis is commonly used as a valid method and a simple assay for evaluating the scavenging activity of antioxidants, since the radical compound is stable and does not have to be generated as in other radical scavenging assays [297]. This method is based on the scavenging of DPPH through the addition of a radical species or an antioxidant that decolourizes the DPPH solution [298]. The results of the antioxidant activity of *Chlorococcum* sp. and *Nannochloropsis* sp. extracts are presented in Tables 7-1 and 7-2. The antioxidant activity of *Chlorococcum* sp. was expressed in % antioxidant activity (AA); the highest inhibition was 73 ± 4 %, which was 65 ± 0.8 mg AAE/100 g of dry weight in the modified BG-11 medium supplemented with KCl 1% (w/v). The highest antioxidant activity of *Nannochloropsis* sp. was 61 ± 2 %, which was 58 ± 0.4 mg AAE/100 g of dry weight in the f/2 medium with a concentration of 75 mg L^{-1} of $NaNO_3$. Based on the DPPH analysis, the antioxidant activity of *Chlorococcum* sp. in the modified BG-11 medium supplemented with acetate was relatively lower than with others; these results showed a similar trend to that of *Nannochloropsis* sp. when the cells were also grown in f/2 medium supplemented with acetate. The results showed that the presence of acetate in the medium resulted in low antioxidant activity, and that total phenolic and carotenoid content, and antioxidant activity in both species could be optimized by selecting the medium composition and processing conditions.

7.3.2. Correlation of total phenolic, total carotenoid content and antioxidant capacity

The F and P values in the ANOVA analysis were 9.876 and 0.001 ($\alpha = 0.05$) in *Chlorococcum* sp. and 9.797 and 0.004 ($\alpha = 0.05$) in *Nannochloropsis* sp. These results provided evidence of the existence of a linear relationship between antioxidant capacity and the two explanatory variables (total phenolic and carotenoid content). The correlation coefficient (R^2) value of the multiple regression analysis is shown in Table 7-3. The R^2 value in *Chlorococcum* sp. (0.537) and *Nannochloropsis* sp. (0.662) indicated that, except for phenolic and carotenoid compounds, other components also contributed to the antioxidant capacities in both microalgae. Compounds including polyunsaturated fatty acids, polysaccharides, other pigments, vitamins and proteins also contribute to the antioxidant capacity in microalgae [285, 299, 300]. The phenolic and carotenoid content might not be a major contributor to the antioxidant capacity of *Chlorococcum* sp. In contrast to *Nannochloropsis* sp., carotenoid content ($P = 0.037$; $\alpha = 0.05$) contributed to antioxidant capacity, but phenolic compounds were not a major contributor to antioxidant capacity in this species. Even phenolic compounds can be antioxidant

compounds in many plant species including vegetables, fruit and medicinal plants [301, 302]. The compounds responsible for their antioxidant activity could be attributed to other molecules in *Chlorococcum* sp. and *Nannochloropsis* sp. It is well known that carotenoids act as antioxidants [208, 285, 303]. A number of papers reported that a significant correlation was demonstrated between antioxidant activity and the phenolic content of microalgae [75, 289, 291, 304, 305]. However, other studies showed contrasting results with no or only a weak correlation between antioxidant activity and total phenol content [291, 292].

Table 7-3. Correlation of total phenolic and total carotenoid content versus antioxidant capacity using multiple regressions analysis.

Species	Coefficient	Standard Error	R^2	t-Stat	P-value
Chlorococcum sp.					
Intercept	26.564	3.714		7.160	$1.6x10^{-6}$
X variable:			0.537		
Total phenolic (X1)	0.051	0.046		1.120	0.278
Total carotenoid (X2)	0.283	0.150		1.886	0.076
Nannochloropsis sp.					
Intercept	18.998	4.230		4.491	0.001
X variable:			0.662		
Total phenolic (X1)	-0.009	0.048		-0.193	0.851
Total carotenoid (X2)	0.372	0.099		3.772	0.037
Y variable (Antioxidant capacity)					

7.4. Summary

The antioxidant capacity, total phenolic content and total carotenoid content of two microalgae were evaluated. Medium composition influenced antioxidant capacity, and total phenolic and carotenoid content. Based on the DPPH analysis, *Chlorococcum* sp. and *Nannochloropsis* sp. showed antioxidant activity. Phenolic compound was not a major contributor to the antioxidant capacity in both species, whereas *Nannochloropsis* sp. showed that carotenoid content contributed to the antioxidant capacity.

CHAPTER 8
Modeling of Growth and Lipid Production in *Chlorococcum* sp.

8.1. Introduction

A mathematical model can be used to analyze and predict the growth and the quantity of product, and help improve the production process in microorganisms including protease production by *Bacillus circulans* [306], ribonucleic acid fermentation by *Candida tropicalis* [307] and lipid production in microalgae [308, 309]. Most microorganism growth processes are explained using a Monod or logistic equations; a logistic equation allows a simple calculation of the fermentation parameters of biological and geometrical significance using sigmoid profiles independent of substrate concentration [306]. In the present study, a logistic method and Leudeking-Piret equations were used for the growth models and the models of lipid production in *Chlorella minutissima* and *Chlamydomonas rainhardtii* [309, 310].

8.2. Model development

Chlorococcum sp. was used in this model; cells were grown on acetate 0 and 70 mM for 14 days of cultivation. The growth was modeled using a Monod equation [306, 307, 309], whereas a Luedeking-Piret equation [311] was used to model lipid production.

8.2.1. Microalgae growth

The following Monod equation is given by:

$$\frac{dX}{dt} = \mu_{max}\left(1 - \frac{X}{X_{max}}\right)X \qquad (1)$$

where $\frac{dX}{dt}$ is the rate of microalgae growth (g L^{-1} h^{-1}); μ_{max} is the specific maximum growth rate (h^{-1}); X is the concentration of microalgae (g L^{-1}); and X_{max} is the maximum of the microalgae concentration (g L^{-1}).

At the beginning of fermentation, $t = 0$, the initial microalgae concentration is considered as $X = X_0$. Integrating Eq. (1) from t_0 to t gives the formulation:

$$X = \frac{X_0 \, X_{max} e^{\mu_{max} t}}{X_{max} - X_0 + X_0 e^{\mu_{max} t}} \qquad (2)$$

8.2.2. Lipid production

Lipid formation was investigated using the Luedeking-Piret model [311]. Based on the Luedeking-Piret equation the rate of lipid production is:

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \qquad (3)$$

where $\frac{dP}{dt}$ is the lipid formation rate (g L^{-1} h^{-1}); α is a lipid formation coefficient (g L^{-1}); and β is a non-growth correlation coefficient (g L^{-1}).

Three kinetic classes of product formation were classified in the fermentation process as follows: Class I – the process in which the product formation appears as a result of primary energy metabolism; Class II – the process in which the product formation arises indirectly from the reaction of energy metabolism; in this type the product results from some side reaction or subsequent interaction between these direct metabolic products.; Class III – the process in which the product formation does not arise from energy metabolism at all, but is rather independently elaborated or accumulated by the cells [312]. For $\alpha \neq 0$ and $\beta = 0$, this process is classified as Class I; there was a linear relationship between product formation and microalgae growth. In Class II ($\alpha \neq 0$ and $\beta \neq 0$), the process of the product formation was complex process where the product is involved many pathways, the product formation was partially connected to microalgae growth. In Class III, the product formation was unrelated to microalgae growth ($\alpha = 0$ and $\beta \neq 0$). In Class II, the Eq. (3) could be simplified as:

$$P = \alpha X + K \qquad (4)$$

where P is lipid concentration (g L^{-1}) and K is the constant of integration.

Using $X = X_0$, $P = P_0 = 0$ ($t = 0$), substituting $\frac{dX}{dt}$ from Eq. (1) into Eq. (3) and integrating, the following expression for lipid production is obtained:

$$P = \alpha X_0 \left(\frac{X_0 \, X_{max} e^{\mu_{max} t}}{X_{max} - X_0 + X_0 e^{\mu_{max} t}} - 1 \right) + \beta \frac{X_{max}}{\mu_{max}} \ln \left(1 - \frac{X_0}{X_{max}} (1 - e^{\mu_{max} t}) \right) \quad (5)$$

8.2.3. Substrate consumption

The substrate requirement was related to the rate of biomass production. The utilization of acetate was modeled using the Luedeking-Piret equation [311]:

$$-\frac{dS}{dt} = \frac{1}{Y_{X/S}} \frac{dX}{dt} + \frac{1}{Y_{P/S}} \frac{dP}{dt} + mX \quad (6)$$

Where $\frac{dS}{dt}$ is the acetate consumption rate (g L^{-1} h^{-1}); S is the substrate concentration (g L^{-1}); t is fermentation time; $Y_{X/S}$ is the yield coefficient of biomass formation; $Y_{P/S}$ is yield coefficient of lipid; and m is a maintenance coefficient (g $g^{-1}h^{-1}$). Using $X = X_0$, $P = P_0 = 0$, $S = S_0$ $(t = 0)$ substituting $\frac{dX}{dt}$ from Eq. (1) into Eq. (6) and integrating, the following expression for substrate utilization is obtained:

$$S = S_0 - \left(\frac{1}{Y_{X/S}} + \frac{\alpha}{Y_{P/S}} \right) \left(\frac{X_0 \, X_{max} e^{\mu_{max} t}}{X_{max} - X_0 + X_0 e^{\mu_{max} t}} - X_0 \right) - \left(m + \frac{\beta}{Y_{P/S}} \right) \frac{X_{max}}{\mu_{max}} \ln \frac{X_{max} - X_0 + X_0 e^{\mu_{max} t}}{X_0} \quad (7)$$

where S_0 is the initial substrate concentration (g L^{-1}) of acetate.

8.2.4. Numerical methods

MATLAB/Simulink was used to solve the proposed model and estimate and evaluate the significance parameter.

8.3. Results and discussion

Modeling using MATLAB/Simulink can provide important answers regarding parameters of product formation. Constructing a model for a fermentation process requires the basic information of the growth of the microorganism, the pattern of medium consumption, the thermodynamic properties and product formation, especially in regard to biomass concentration, growth rate, substrate consumption and metabolic products. The Simulink model is shown in Fig. 8-1.

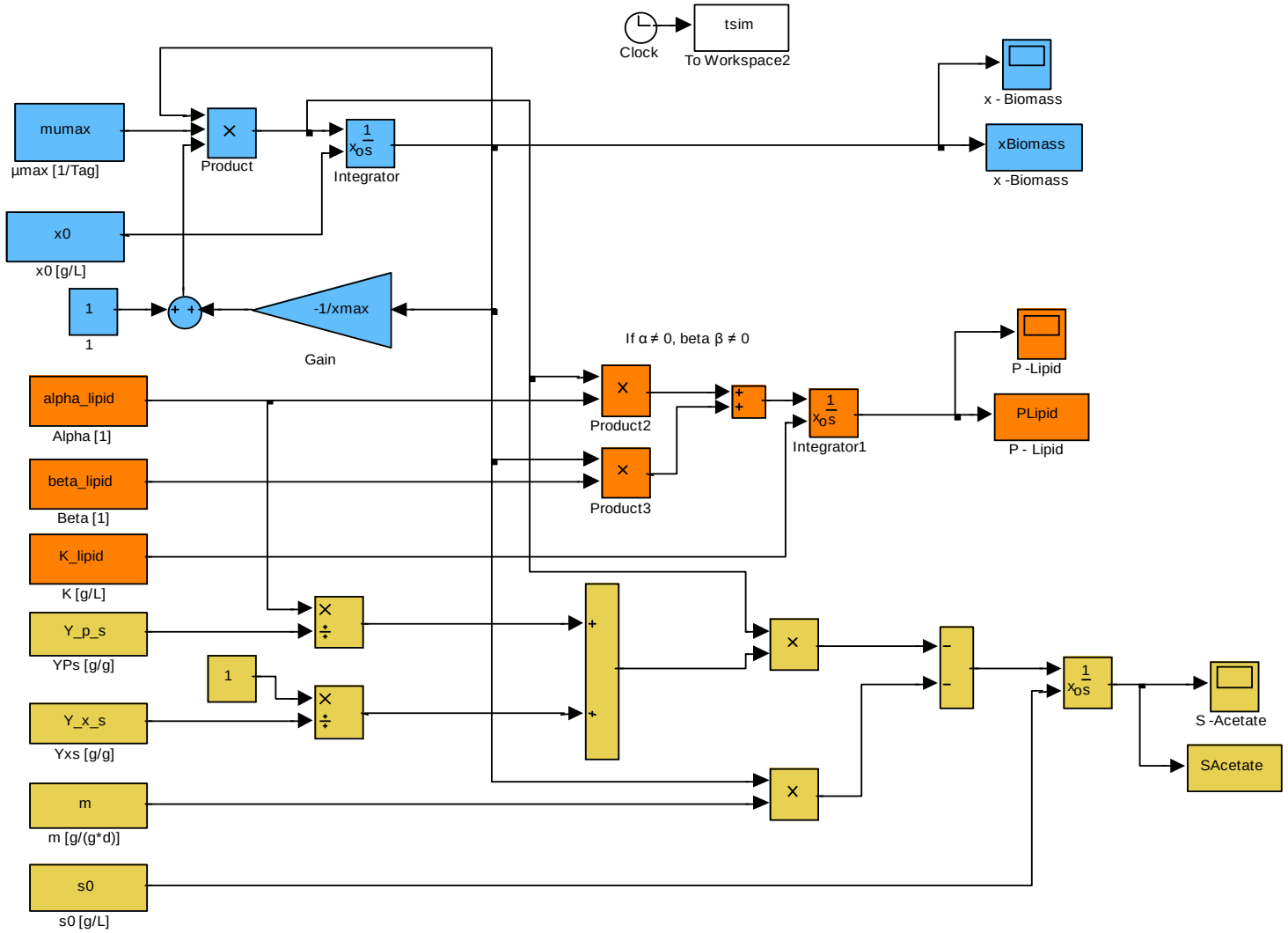
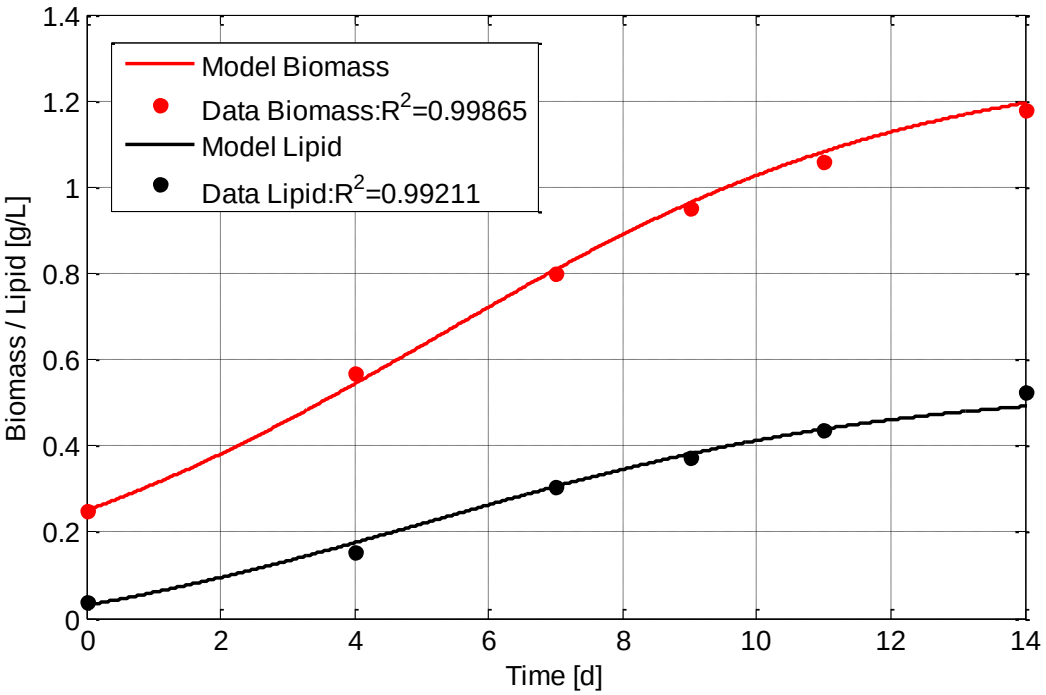


Fig. 8-1. The Simulink model for parameter estimation of biomass, lipid production and substrate consumption.



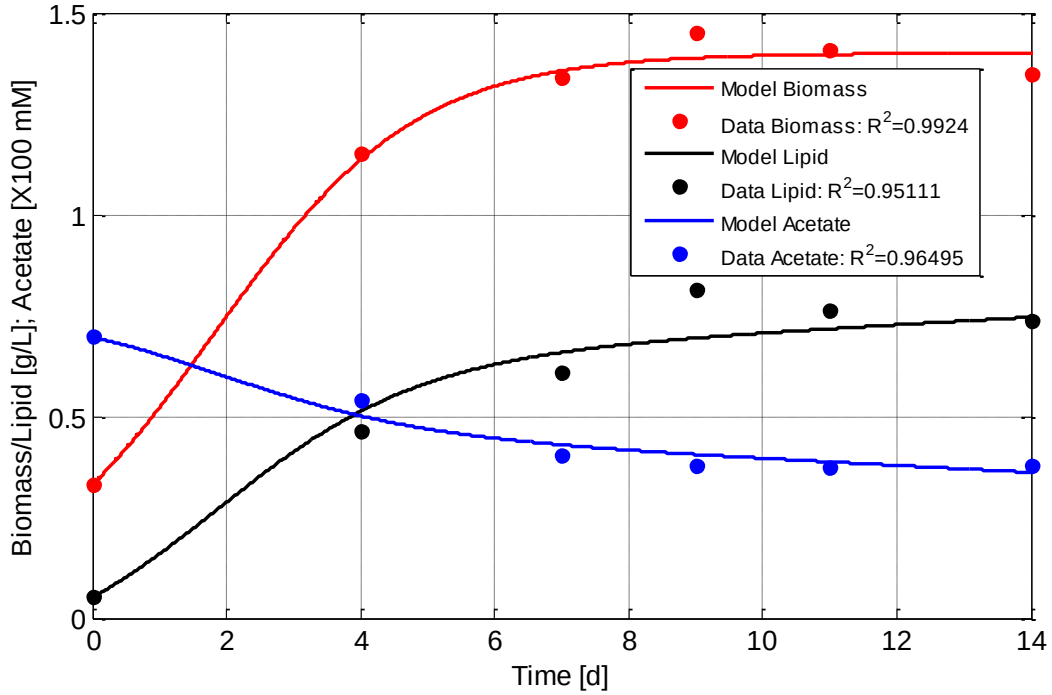


Fig. 8-2. Comparison of experimental data and model of biomass, lipid and acetate consumption by *Chlorococcum* sp.

Table 8-1. The various parameters that were used in this model.

Parameter	Acetate concentration (mM)	
	0	70
Biomass		
μ_{max} (day^{-1})*	0.276	0.657
X_0 ($g\ L^{-1}$)*	0.25	0.33
X_{max} ($g\ L^{-1}$)	1.3	1.4
R^2	0.9986	0.9924
Neutral lipid production		
α ($g\ L^{-1}$)	0.5	0.55
β ($g\ L^{-1}$)	0.001	0.0065
K ($g\ L^{-1}$)	0.03	0.05
R^2	0.9921	0.9511
Acetate		
Yx/s ($g\ g^{-1}$)	-	5
Y_P/s ($g\ g^{-1}$)	-	5
m ($g\ g^{-1}d^{-1}$)	-	0.006
R^2	-	0.9649

* Experimentally calculated parameters and those used in the model

Growth of *Chlorococcum* sp., lipid production and acetate consumption were modeled in Fig 8-2 and the parameters used are shown in Table 8-1. As shown in Fig 8-2, the experimental data describing the biomass concentration correlated well with the model and the correlation coefficients (R^2) in cultivation with acetate 0 mM and 70 mM were 0.9986 and 0.9924, respectively, which suggested that the fit was excellent. The kinetic process of product formation was classified into three categories. According to Gaden [312], the model was categorized as Class II since the product formation was partially connected to microalgae growth ($\alpha \neq 0$ and $\beta \neq 0$). The values of the correlation coefficients were 0.9921 and 0. 9511 under conditions with acetate 0 mM and 70 mM, respectively, which indicated that the results of the model were reasonable. The Luedeking-Piret model of the substrate utilization fitted with the experimental data. The correlation coefficient was 0.9649, which indicated that the model fitted with the data observation satisfactory.

8.4. Summary

The growth of alga and lipid formation were modeled for *Chlorococcum* sp. under photoautotrophic and mixotrophic conditions. A mathematical model was developed in order to help understand the factors that influence the biomass and lipid production. The optimum values of the mathematical model parameters were determined for logistic and Luedeking-Piret equations. The obtained model showed a satisfactory fit with the experimental results.

CHAPTER 9

Conclusions and Future Research

Two strains of microalgae were studied with regards to biomass concentration, total lipid content and fatty acid content and composition. The strains were a freshwater and a marine microalga, *Chlorococcum* sp. and *Nannochloropsis* sp., respectively. Lipids in both microalgae can be produced under photoautotrophic and mixotrophic conditions. Under photoautotrophic conditions *Chlorococcum* sp. was the most suitable for further studies due to its fast growth and relatively high lipid productivity. Moreover, both microalgae were suited for further studies due to their high biomass production and high lipid productivity, being easy to cultivate and having a relatively high lipid content under mixotrophic conditions. Biomass productivity, total lipid productivity and fatty acid content and composition are limited by several factors, and nutrients play a key role. Both microalgae were able to use carbon dioxide as a carbon source; this provides the possibility of using carbon dioxide from industrial waste gases for growth. Under mixotrophic conditions both cells were able to use acetate and crude glycerol for growth. Acetate and crude glycerol are inexpensive carbon sources; acetate can be obtained from anaerobic digestion and crude glycerol can be obtained from biodiesel production. The cultivation of both microalgae under mixotrophic conditions resulted in higher growth rate, cell density and lipid content. In addition, a two-stage strategy could be used to enhance lipid productivity in *Chlorococcum* sp. A two-stage strategy with a sufficient initial nutrient level followed by a nutrient starvation strategy could be addressed for rapid and high biomass generation with a high lipid productivity. *Chlorococcum* sp. showed good results under a two-stage strategy. Therefore, this strategy can be applied to *Nannochloropsis* sp. for further research. Moreover, palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) are the major components of both microalgae. Fatty acids, including saturated and unsaturated types, have several functions; triaclyglycerols have antimicrobial and anti-inflammatory properties and are beneficial for skin care [244]; polyunsaturated fatty acids, linoleic acid and α-linolenic, are essential to the human metabolism and help prevent certain medical conditions such as coronary heart disease, inflammation, and autoimmune

deficiency, etc. [313]; oleic acid, linolenic and palmitic acid also show antimicrobial activity [59]. It is known that palmitic acid and oleic acid are used in the biodiesel industry. Due to their properties in both microalgae, these microalgae show promise as a source of bio products that can be applied in various industries such as the food, pharmaceuticals, cosmetics as well as energy industry. Therefore, further research is required to investigate the abilities of both microalgae regarding antimicrobial activity.

The ethanolic and aqueous extracts of *Chlorococcum* sp. and *Nannochloropsis* sp. produce anti-oxidative compounds that have the effect of inhibiting DPPH radicals. These microalgae produce natural antioxidants which can be used in the food, pharmaceutical as well as cosmetic industry. However, the compounds responsible for the antioxidant activity of *Chlorococcum* sp. and *Nannochloropsis* sp. extracts are not yet known. Therefore, further studies are required to identify the antioxidant compounds and a culture medium is needed to optimize the productivity of antioxidants. Moreover, a detailed analysis of carotenoids is required in order to determine which carotenoid types belong to both microalgae.

The modeling of cell growth, lipid production and substrate consumption in *Chlorococcum* sp. showed a satisfactory fit to the measured data; the product formation was a complex process and was partially connected to microbial growth. The findings suggested that the developed model could be effectively used for the design, control and scale-up of microalgae production. The developed model could be applicable for predicting growth and lipid production in other microalgae species. However, the testing model is recommended to be used before the fermentation process.

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Appendixes

Appendix A

Analysis of acetate in BG-11 medium

Medium	Day / Acetate concentration mM				
	0	3	6	8	10
M2 (Low light)	77.04 ± 2	70.10 ± 2	68.91 ± 1	54.87 ± 1	18.79 ± 0
M3 (Low light)	75.35 ± 1	72.98 ± 3	39.62 ± 1	21.33 ± 1	16.25 ± 1
M2 (Medium light)	74.42 ± 3	74.74 ± 2	68.79 ± 2	67.16 ± 2	70.20 ± 1
M3 (Medium light)	76.82 ± 2	74.82 ± 1	77.18 ± 3	71.42 ± 1	75.25 ± 3
M2 (High light)	68.12 ± 1	78.84 ± 3	78.82 ± 2	75.82 ± 2	77.95 ± 1
M3 (High light)	67.59 ± 1	78.50 ± 1	79.00 ± 3	78.12 ± 1	81.83 ± 2

Appendix B

Analysis of nitrate in f/2 medium

Medium	Day / Nitrate concentration (mg L^{-1})						
	0	2	3	5	6	9	10
f/2 25 mg L^{-1} $NaNO_3$	14.8 ± 1	0	0	0	0	0	0
f/2 75 mg L^{-1} $NaNO_3$	51.8 ± 2	15.1 ± 2	0	0	0	0	0
f/2 150 mg L^{-1} $NaNO_3$	103± 1	78 ± 3	55 ± 1	39 ± 2	33 ± 1	28 ± 2	22 ± 1

Analysis of nitrate in BG-11medium

Medium	Day / Nitrate concentration (mg L^{-1})			
	0	2	4	6
Ac2	14.8 ± 1	0	0	0
Ac4	15.9 ± 0	0	0	0
Ac6	15.3 ± 0	0	0	0
Ac8	18.8 ± 1	0	0	0
RG4	15.7 ± 1	0	0	0
RG10	16.9 ± 0	0	0	0
RG20	17.0 ± 1	0	0	0
RG20C	15.7 ± 0	0	0	0
RG30	17.6 ± 2	0	0	0

Ac2: Acetate 2 g L^{-1}

Ac4: Acetate 4 g L^{-1}

Ac6: Acetate 6 g L^{-1}

Ac8: Acetate 8 g L^{-1}

RG4: Crude glycerol 4 g L^{-1}

RG10 : Crude glycerol 10 g L^{-1}

RG20 : Crude glycerol 20 g L^{-1}

RG20C : Crude glycerol 20 g L^{-1} and aerated with CO_2

RG30 : Crude glycerol 30 g L^{-1}

Appendix C

Analysis of crude glycerol in f/2 medium

Medium	Crude glycerol ($g\ L^{-1}$)	Day / Gycerol concentration ($g\ L^{-1}$)				
		0	3	6	8	10
RG4	4	0.056 ± 0.001	0.087 ± 0.022	0.091 ± 0.001	0.089 ± 0.009	0.110 ± 0.002
RG10	10	0.138 ± 0.003	0.231 ± 0.012	0.247 ± 0.005	0.251 ± 0.012	0.273 ± 0.011
RG20	20	0.239 ± 0.001	0.510 ± 0.001	0.528 ± 0.010	0.567 ± 0.009	0.630 ± 0.017
RG20C	20	0.258 ± 0.006	0.491 ± 0.081	0.498 ± 0.013	0.500 ± 0.015	0.484 ± 0.022
RG30	30	0.405 ± 0.005	0.473 ± 0.024	0.570 ± 0.021	0.559 ± 0.021	0.483 ± 0.018

Appendix D

Analysis of acetate in f/2 medium

Medium	Day / Acetate concentration ($g\ L^{-1}$)					
	0	2	3	6	8	10
Ac2	1.933 ± 0.01	1.756 ± 0.08	1.814 ± 0.05	1.427 ± 0.05	1.642 ± 0.04	1.406 ± 0.08
Ac4	3.939 ± 0.02	4.087 ± 0.10	4.094 ± 0.10	3.935 ± 0.12	4.043 ± 0.02	3.802 ± 0.09
Ac6	6.307 ± 0.22	6.629 ± 0.04	6.531 ± 0.33	7.323 ± 0.17	7.207 ± 0.13	7.197 ± 0.13
Ac8	8.192 ± 0.15	8.122 ± 0.23	8.564 ± 0.43	8.944 ± 0.56	8.120 ± 0.21	8.256 ± 0.33

Appendix E

Graphic of standard solution of ascorbic acid